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MSS. for No. 2 of Vol. 19 should be in the hands of the Editor before March 1st, 1925. Contributors are specially requested to arrange references in the manner uniformly adopted in this Journal.

OBITUARY NOTICE.

WILLIAM MADDOCK BAYLISS.

1860–1924.

No physiologist of recent times has left so important a mark on the progress of his science as Sir William Bayliss. There is none whose untimely death will be so deeply regretted, and whose place it will be so hard to fill. As one who has known him throughout his scientific career it has fallen to my lot to pen a few brief words of appreciation and sympathy. More I cannot attempt: fuller biographies of his life and work will doubtless appear elsewhere.

His life was devoted whole-heartedly to research; this was carried out at Oxford, at University College, London, and in his private laboratory at his Hampstead home. His energy was untiring, his writings numerous, his discoveries important and his influence on physiological science far-reaching and world-wide.

His name will go down to posterity as the apostle of "General Physiology" and for his monumental treatise on that subject. Bayliss, however, was in truth a general physiologist, not in the narrower sense of that term, but in its wider or literal meaning. The tendency of the modern teacher is specialisation, but Bayliss had none of this; there were few or no aspects of his science which he neglected or failed to illuminate. He made no pretence of being a chemist; yet his selection as joint editor of this *Journal* was universally hailed as the wisest choice that could have been made, and there is nobody who will suffer by his death more than those responsible for the conduct of the *Biochemical Journal*; his biological instincts, his catholic attainments and wide knowledge make his loss irreparable; in like manner he never posed as a physicist or a mathematician, but again he of all others called these sciences in an effective way into the service of the science of life.

His all-embracing sympathies with the learner made him throughout a guide, philosopher and friend to all enquirers, and these will keenly feel the gap now left.

I have seen it stated that to the beginner and average student he was somewhat indifferent, but if this were so (and I am by no means sure that it was so), I am certain that it was due to no lack of fellow-feeling but because he had a higher purpose to fulfil, namely to guide those with more ambitious aims, and especially to instruct his fellow-physiologists.

His life as a University Professor was happily untrammelled by work of a purely administrative nature, though again he had great business gifts, as was proved by the high place he occupied in the councils of his fellows, and his effective chairmanship of important committees of investigators.

His original and lucid way of looking at the problems before him was his great asset; a striking example is furnished by the fact that he was the earliest to recognise the important part which Physical Chemistry was to play in the progress of modern physiology, and in the physiology of the future.

When stricken less than two years ago by an obscure disease which was difficult to diagnose and the progress of which none could stay, his numerous friends followed with anxiety the reports of its fell progress; and when the dreaded end came, one and all felt not only the great loss they had sustained, but realised they had to mourn the departure of a truly great man.

To his immediate relatives and especially to his sorrowing widow and children these feelings will be mingled with others of a more personal nature, and to them one can only offer deep and respectful sympathy.

W. D. H.

CLIV. THE CONSTITUENTS OF THE SAP OF THE VINE (*VITIS VINIFERA* L.).

By ARTHUR WORMALL.

The Department of Physiology and Biochemistry, University of Leeds.

(*Received June 28th, 1924.*)

In a study of the sap exuding from the cut vine, Priestley and Armstead produced evidence in support of the view that besides mineral salts, there are present organic substances which are directly responsible for the production and maintenance of the necessary osmotic pressures. In view of the importance of these organic substances with regard to the alteration in physico-chemical equilibria and to the nutritive material supplied to the upper parts of the plant, it was considered desirable that a more exhaustive study of the constituents of the sap should be made, and for this purpose, a large quantity of the sap from bleeding vines was collected and examined as described below. The results obtained furnish information with regard to the nature of the organic and inorganic substances present, and in many cases those which are absent; they do not support the old idea that the sap is merely a dilute solution of inorganic salts.

Very little information could be obtained from previous investigations on the sap of plants, these investigations either having been carried out when the methods of chemical analysis were not sufficiently accurate to render the results conclusive, or else having been limited to the determination of the amounts of organic and inorganic matter present, and in some cases, the amounts of sugar exuded under varying conditions. In comparing the sap contents of the birch and maple, Schroeder [1869] estimated the percentage of sugar present, and indicated the variations in the amounts of calcium, magnesium, potassium and phosphoric acid present in different parts of the stem and roots. More recently, Moreau and Vinet [1923] have made analyses of the "bleedings" of the vine over varying lengths of time, and during and after periods of frost. These authors found that if the temperature is maintained at zero, or a little below, maximum richness results, though the volume exuded diminishes, and, over this period, the organic matter may form 94 % of the total solid matter, and of this organic matter 91·3 % is in the form of reducing substances. Thus they state that during a period of frost, followed by a rise of temperature, the sap yielded is very rich in organic material, and they refer to the fact that in Canada the sap of *Acer saccharinum* is collected in preference on a cold, frosty day, the liquid obtained being then much sweeter than at other times, and containing up to 35·7 g. of saccharose

per litre. This view, however, is opposed to that of Wiegland [1906], who states that the pressure and flow in the birch and the grape depend very little on the temperature of the air. Wiegland classifies trees into two groups, those which bleed early in the season, *e.g.* the maple, and those which, like the birch and grape, bleed later in the spring, and he attributes the flow of sap in the latter group entirely to root pressure. The later work of Moreau and Vinet shews that the vine behaves like the maple with regard to temperature variations, and this view is supported by the observations made during the collection of the vine sap for the work described here. The results of the present investigation are in entire disagreement with the statement made by Wiegland that the sap of the grape is almost pure water and contains no sugar.

From the "bleedings" of several vines, Moreau and Vinet obtained results which are expressed in tabular form as the mean volume collected in 24 hours, the dry matter at 100°, the ash, reducing substances (expressed as glucose) and the other organic matter. The figures given in this table prove that the view that the liquid contains 1–2 g. dry matter per litre, of which approximately two-thirds is organic and the rest mineral matter, is not strictly correct, and the authors conclude that the constituents vary between wide limits, the variations being related to a large extent to the external temperature. When, however, the sap is collected over a long period and the average figures for the whole period calculated, as was done in the work described in this paper, the older generalisation is found to be fairly accurate; the sap collected was found to contain 1·56 g. of solid matter per litre, of which 0·56 g. was inorganic and 1·00 g. organic. No distinction has been drawn between the sap yielded at the commencement of bleeding and that exuded later, and thus the results represent the total amounts of the various substances translocated by the xylem sap, while the percentages calculated represent the average figures for the whole period of bleeding. Quantitative tests, not recorded below, supported the results obtained by Priestley and Armstead, who found that the sap yielded in the later periods of bleeding contained less organic matter than the first bleedings. In this connection, it is interesting to note that Schroeder [1869] found that the amount of sugar present in the sap of the birch and maple decreased as bleeding proceeded.

From the physiological view-point, though not in a quantitative sense, the most important substances present appear to be the sugars, and a considerable time was spent in the estimations of the various carbohydrates. Priestley and Armstead [1922] found that hexoses and a much smaller amount of a disaccharide, presumably cane-sugar, were present, and this is confirmed by the results quoted below. Further, by the application of various methods of sugar estimation, it was found, as far as the methods adopted would allow, that the reducing sugars are glucose and fructose, the disaccharide cane-sugar, and that no other sugars are present with the possible exception of a trace of galactose. No polysaccharide could be detected, while disaccharides other than cane-sugar appear to be absent.

These results agree in substance with those obtained by Dixon and Atkins [1916] who found that the sap of various trees contained both reducing sugars and cane-sugar, and that during the vernal mobilisation of the reserves, hexoses and maltose are present, while the latter is absent at other times. These authors attribute the whole of the rise in osmotic pressure, as the higher levels of the tree are reached, to the sugars present, while admitting that other organic matter may be present, and their results are generally in entire agreement with this view. In certain of the examples given, however, a rise in the osmotic pressure due to non-electrolytes is not accompanied by a parallel rise in sugar content, and here the factor concerned may be the "other" organic matter, for which no figures are given. The fact that cane-sugar is the most important sugar in translocation and that maltose is absent in the majority of cases, as in the vine, appears worthy of further consideration with a view to the study of the possibility of conversion of glucose into fructose and the subsequent formation of cane-sugar.

The other organic matter present consists mainly, if not entirely, of the dibasic acids, oxalic, tartaric, malic and succinic acids, present as their calcium salts, and the total amount of these acids present exceeds that of the sugars. Schroeder [1869] records the presence of malic acid in the sap of both maple and birch.

The inorganic matter was found to consist of the chlorides, sulphates, phosphates, nitrates and nitrites, and silicates of sodium, potassium, calcium, iron, magnesium, and to a less extent manganese and aluminium. This is in close agreement with the view of Jost that Cl, S, P, Si and the metals K, Na, Ca, Mg and Fe occur regularly in appreciable amounts in the ash of all plants and that Al and Mn are usually demonstrable. The iron was present in a readily dialysable form and thus is not in the form of a colloidal complex.

The whole of the nitrogen present appears to be in the form of nitrate and nitrite, with the exception of a trace of organic nitrogen associated with the enzymes. The most likely explanation of the absence of organic nitrogen is that the presence of nitrites and amino-acids in an acid solution is incompatible. Vine sap which had been stored in a coloured bottle in a dark cool cellar contained a very small amount of free amino nitrogen as measured by formaldehyde titration (equivalent to 0·45 cc. of $N/10$ NaOH per 100 cc. of sap).

No substance of fat-like nature could be detected in any appreciable quantity, though traces of a neutral fat and a fatty acid were isolated from the concentrated solution after removal of the salts, sugars, etc. by dialysis, and also from the precipitate obtained with lead acetate. The amounts obtained, however, were too small to allow of any tests other than those of solubility and staining, and they would appear to have no great significance in view of the extremely low concentration.

It was thought possible that certain "soluble" or fairly diffusible enzymes might be present and tests were carried out for some of them. Those found

to be present were peroxidase, diastase and a small amount of catalase, while those absent were invertase, maltase, lipase, glycerophosphatase, proteases and rennin. These results appear to agree in substance with those obtained by other workers on tree sap, and since the tests described below were carried out, it has been noted that Palladin and Manskaja [1923] have identified peroxidase and Votchal [1916] has discovered oxidases and diastase in tree sap. Priestley and Swarbrick [1924] have shown that peroxidase, diastase, and catalase are present in the sap of the sycamore, and have also identified invertase (unpublished observation). With regard to the oxidising enzyme present, it is interesting to note that no peroxide could be detected, since the vine juice would not blue guaiaconic acid or benzidine solutions, except on the addition of H_2O_2, and this absence of peroxide could be demonstrated with solutions which had been preserved with toluene and exposed to the air for eight or nine months.

Method of collecting the sap and of its treatment prior to analysis.

Previous attempts to obtain a sterile juice had not proved entirely satisfactory, and it was considered desirable that more stringent precautions should be taken to ensure the absence of active bacterial organisms. The sap obtained by merely cutting the stem, attaching a piece of glass tubing to the latter by means of rubber tubing, and collecting the liquid under toluene, was found to turn cloudy after exposure to the light for several hours. This cloudiness, which was much more pronounced in the absence of toluene, was regarded as an indication of bacterial action, but, as is mentioned below, there is a phenomenon of preliminary opalescence which is not associated with ordinary bacterial action. Because of the tendency for bacterial action to take place, and as a considerable time elapses before the juice can be prepared for chemical analysis, the cut surface of the stem, the knife used for trimming the stem, and the different attachments were all carefully sterilised.

Each vine stem was sawn across about 1 to 2 ft. from the ground, the saw being previously sterilised in the flame, and the outside fibrous bark stripped off, great care being necessary to prevent injury to the phloem. The cut surface was then trimmed with a sterilised knife and the stem and the cut surface wiped with an alcoholic solution of K_2HgI_4. The metal cap, a turned iron cylinder about 2 inches in length with a side tube attached to a short length of composition tubing, was sterilised by immersion in boiling water, and the rubber collar, a short length of the inner tube of a motor-cycle tyre, washed with alcoholic K_2HgI_4. A pad of cotton wool soaked with toluene was fitted in the top of the metal cap. The rubber collar was then slid over the stem, and the whole bound on with wire and adhesive antiseptic surgical tape. The sap was collected in Winchester quart bottles previously sterilised in steam and containing 15–20 cc. of toluene, and as soon as one bottle became full, it was replaced by another containing toluene as before. The displaced air passed out of the bottle as the sap collected through a second

tube which was bent so that the free end pointed downwards, and was closed with cotton wool. The rubber stopper carrying the two tubes was in position when the bottle was sterilised in the steam steriliser. Fig. 1 illustrates the mode of collection of the sap.

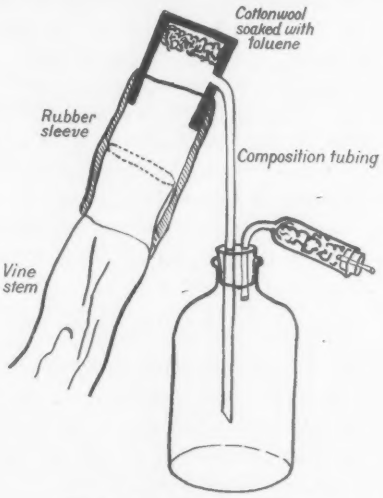

Fig. 1. Apparatus in position.

The liquid collected in this way was removed from the glasshouse as soon as possible and stored in a dark cool cellar until required. Some of the samples, usually the first "bleedings," were water-clear, but others were distinctly opalescent, although no bacteria could be detected by the ordinary bacteriological tests. Priestley and Raper had both thought that light played an important rôle in this change, and the latter investigated the action of rays of sunlight on some of the clear solution, setting up a control of the same liquid in a tube covered with lead foil on the outside. The solution exposed to the light rapidly became cloudy, while the control remained unchanged.

Concentration of the sap.

The dilute sap was concentrated by distillation *in vacuo* from three one-litre glass distillation flasks at a temperature of 50–55° and a pressure of 20–30 mm. Whenever the distillation was stopped, toluene was added to the concentrated juice, and as a precautionary measure, the air bubbled through the liquid to assist distillation was filtered through cotton wool. As the liquid was concentrated, a brownish-grey deposit separated on the sides of the distilling flasks, and this increased in bulk as distillation proceeded. Finally, when 90,300 cc. of sap had been concentrated to about 1 litre, the distillations were stopped, the solutions filtered and the precipitate washed with a small

quantity of distilled water. Some of the precipitate which adhered to the sides of the flasks was dissolved in a small quantity of warm dilute hydrochloric acid and treated separately. The main bulk of the precipitate (ppt. 1) was then dried on a porous plate in a vacuum desiccator, weighed and examined later, while the solution, which had a volume of 1205 cc. and to which was added 15–20 cc. of toluene, was stored in an ice-chest during the whole period of examination. When this solution had stood for a few days, it was found to contain a small amount (0·45 g.) of a crystalline deposit (ppt. 2) which, after removal by filtration, was found to consist of colourless prisms. The filtrate from this deposit, henceforward referred to as the conc. vine juice, had a slight brownish-yellow colour, and prepared and stored in this way, was found to be free from active bacterial organisms.

Dr H. V. Phelon, of the Department of Bacteriology at this University, kindly examined the solution at intervals and found no vegetating organisms present at any time.

General method of procedure.

The distillate obtained during the concentration of the juice was tested for volatile acids, reducing substances, and alcohol. No precipitate could be obtained by adding lead acetate, silver nitrate, or barium chloride solution to the distillate, and no reducing substances could be detected. By redistilling about two litres of the distillate, collecting only the first tenth and distilling off the first tenth of this last distillate, a solution was obtained which contained a small amount of alcohol, but no reducing substances. The alcohol was identified by the formation of iodoform and ethyl benzoate, both detected by smell, and also by the reaction with nitric acid and potassium dichromate. The amount of alcohol present, even after concentration by this method was too small to be of any significance, and it is considered most probable that the source of it was the spirit used for sterilising the instruments, etc.

The various deposits, ppt. 1, the hydrochloric acid solution of part of the same ppt., and ppt. 2 were separately examined for inorganic and organic substances and their ash and phosphorus contents determined.

The main bulk of the concentrated solution was first examined for sugars by the methods described below. The solution was first cleared by the addition of basic lead acetate until maximum precipitation was produced. This method of clearing the solution and of removing organic acids which would affect the polarimetric readings, appears to be the only one available, but in spite of the statement to the contrary made by Davis and Daish [1913], it does not appear to be entirely satisfactory. Adsorption of sugars by the precipitate does take place to some extent and the adsorbed sugar cannot be removed by washing with water. Thus the precipitate obtained with basic lead acetate was found to contain fructose and possibly glucose, even after repeated washing with water, and although the amounts were not considered sufficient to vitiate the results obtained, preferential adsorption of one sugar may in some cases

lead to erroneous views. Qualitative tests such as the preparation of the osazone, reactions with Seliwanoff's reagent, etc., were carried out with the solution and quantitative estimations of the reducing power and polarimetric value were made. The disaccharides were estimated by the alteration in reducing power and rotation by hydrolysis with HCl, citric acid, and also a solution of invertase. The results, which were almost identical by the three methods, indicated the presence of a small amount of cane-sugar, and the absence of other hydrolysable carbohydrates. The other methods used for the various sugar estimations are described later.

The precipitate produced on adding basic lead acetate to the concentrated vine juice was washed with water, dried on porous plates in a vacuum desiccator and weighed. After being ground up to a paste with water, it was decomposed with H_2S, and the filtrate from the precipitated PbS examined for various organic and inorganic acids and polysaccharides, and both filtrate and precipitate were examined for fat-like substances.

About 100 cc. of the concentrated vine juice contained in a very permeable collodion sac were dialysed under a slight pressure against running water and the non-dialysable portion examined for proteins, carbohydrates and fats. As was expected, practically all the solid matter passed through the membrane and the solution left in the sac contained merely the enzymes, together with a very small quantity of both a neutral fat and a fatty acid.

For the different enzyme tests described later, various solutions were used, these being the ordinary dilute sap, the concentrated juice, dialysed dilute sap, and dialysed concentrated juice, and whenever it was thought desirable, suitable co-enzyme solutions were added to the dialysed solutions in order to replace any lost by dialysis.

Tests for organic nitrogen by the fusion with sodium method were carried out with all the precipitates and also with the residues obtained by heating to dryness on a water-bath the dilute sap, and also the concentrated vine juice. In some cases, the reactions were very faintly positive, but were thought to be due to the enzymic substances present, and at other times to reactions between nitrites and nitrates and the organic matter.

Estimations of the solid content of the concentrated juice were made by heating on a water-bath to dryness, drying at 100° and weighing, while the ash contents of this residue, and of the other precipitates, were determined by heating in an electric furnace at 500-550° until constant weight was attained.

First deposit (ppt. 1).

This deposit which, together with that which had to be dissolved in dilute HCl, contained 40·1 g. of solid matter, had a total ash content of 14·55 g. in the form of the phosphates and oxides of calcium and iron, and to a less extent magnesium. Small amounts of silica and alumina, and a trace of manganese were also present. Over half the precipitate was soluble in dilute acetic acid, and this acetic acid solution was examined for the calcium salts of certain

organic acids. The acids present proved to be tartaric, malic and succinic, the first being precipitated as its calcium salt on neutralisation of the solution, and the last two as calcium salts on addition of excess of alcohol to the filtrate. Tartaric acid was identified by the colour reaction of Pinerua [1897] with β-naphthol and conc. H_2SO_4, by the reaction with conc. H_2SO_4 and resorcinol, by the formation of potassium bitartrate, and by a M.P. determination. Similarly the succinate and malate were decomposed with acid, but in these cases the acids were extracted by continuous ether extraction and identified as in the lead acetate ppt.

That portion of ppt. 1 not soluble in dilute acetic acid was extracted with dilute HCl, when over half the remainder dissolved and could be reprecipitated by neutralisation with NaOH. This ppt. proved to be calcium oxalate and the acid was isolated by decomposition of the ppt. with the calculated amount of H_2SO_4 and also by ether extraction of an acid solution of the ppt.

After extraction with dilute acetic acid and dilute HCl there still remained some insoluble matter in ppt. 1, and this was found to be a mixture of silicates, and a small amount of coagulated protein matter presumably from the enzyme matter destroyed during part of the distillation process. Thus ppt. 1 consists of the oxalates, tartrates, malates, succinates, phosphates and silicates of calcium, iron, magnesium, and aluminium, with a very small amount of coagulated protein matter. The bulk of the ppt., at least 80 %, consists of the salts of the organic acids with Ca and Fe, and the acids are present in roughly equal quantities. From an acid solution of ppt. 1, a mixture of organic acids was obtained by continuous ether extraction, and from this mixture an acid with a melting-point of 161–2° was isolated. The sharpness of the M.P. indicated that the substance was fairly pure and presumably not tartaric acid, but sufficient was not available for identification. This acid was very soluble in water, but only slightly soluble in ether.

Second deposit (ppt. 2).

This small deposit of 0·45 g. was found to consist of calcium tartrate, together with a very small amount of either malate or succinate. Almost the whole of the precipitate consisted, however, of the tartrate, and this acid was identified by the methods described in the previous chapter.

The precipitate with basic lead acetate.

The precipitate obtained by adding excess of basic lead acetate to 200 cc. of concentrated vine juice was washed well with water, dried on porous plates *in vacuo*, and weighed 24·2 g.

The bulk of this precipitate was made into a paste with a small quantity of distilled water, more water added, and the lead salts decomposed by H_2S. After removal of the precipitated PbS by filtration, the solution was evaporated to a small bulk under reduced pressure. This concentrated solution containing

traces of phosphoric, sulphuric and hydrochloric acids, and a large amount of organic acids, was then shaken up six times with an equal volume of ether. The mixed ethereal extracts and the aqueous solution remaining after ether extraction were examined separately for organic acids, using, in the first place, the differences in the solubilities of the various calcium salts in hot and cold water, and alcohol. The free acids were then separated from their calcium salts by decomposition with dilute H_2SO_4, or whenever possible by ether extraction from a phosphoric acid solution of the calcium salt.

The ethereal extract of the concentrated solution of the acids was found to contain malic acid and a smaller amount of succinic acid. Various solvents were used, but it was found impossible to obtain more than a small amount of malic acid in the crystalline state. This was found to melt at the correct temperature (100°). In view of the difficulty of obtaining malic acid in the pure crystalline condition, and owing to the absence of distinctive colour reactions, it is realised that the criticism levelled by Franzen and Keyssner [1923] against the methods used for identifying malic acid in plants, and against the view that this acid is widely distributed, is quite justifiable, and whenever possible, tests should be carried out to exclude other acids, such as succinic acid, whose calcium salts have properties very similar to those of calcium malate. In the present work, not enough of the precipitate was available to permit use of the method recommended by these authors, and so recourse had to be made to other methods. A small amount of the acid was obtained in the crystalline condition and found to melt at the correct temperature, while the acid could be extracted from an acid solution of the calcium salt by extracting a few times with ether, whereas succinic acid is only very slightly soluble in ether. Finally, the colour reaction with β-naphthol and conc. H_2SO_4 suggested by Pinerua [1897] was obtained by using some of the free acid. This test, used in conjunction with controls in which the same weights of succinic, tartaric, malic, citric, and malonic acids were employed, proved extremely satisfactory and the importance of this test as a confirmatory test, when compared with suitable controls, does not appear to have been realised sufficiently.

The acids not extracted by the ether were found to be succinic acid, a smaller amount of malic acid, and a trace of tartaric acid, together with the inorganic acids, HCl, H_2SO_4 and H_3PO_4. To the neutralised solution, calcium chloride solution was added in excess, and a slight precipitate separated on long standing. This precipitate was identified as calcium tartrate by the tests adopted in connection with the first deposit (ppt. 1). The filtrate, on boiling, gave a further small amount of precipitate which, however, was not calcium citrate but a mixture of the malate and succinate. The solution was filtered and excess of alcohol added, when a bulky white precipitate separated out. This precipitate proved to be calcium succinate with a smaller amount of calcium malate, the two acids being separated by their different solubilities in ether. By this method, a sample of crystalline succinic acid was isolated

which melted at 184–185° (M.P. succinic acid 185°), and had the same melting point when mixed with an equal amount of pure succinic acid. The acid also gave an insoluble iron salt from neutral solutions and this ferric salt on decomposition with dilute NH_4OH and removal of excess of ammonia by boiling, yielded a crystalline precipitate, presumably barium succinate, with 5 % barium chloride solution.

The basic lead acetate precipitate was thought at one time to contain lipins, and possibly the lead salts of the higher fatty acids, but no lipin could be isolated. A very small amount of nitrogen was found to be present by the fusion with sodium method, but this was attributed to traces of adsorbed nitrite and nitrate, for these could be detected in the precipitate. Traces of a fat-like substance could, however, be isolated from the lead acetate precipitate by extraction over an extended period with alcohol, ether or chloroform. The residue obtained by evaporating off all the solvent used was found to be insoluble in water, soluble in all the fat-solvents, and could be stained with Nile-Blue, Sudan III and Scharlach R. Not enough of the fatty substance was available for a M.P. determination, but by means of light petroleum the residue was divided into two portions. That portion more soluble in light petroleum behaved as a neutral fat, and the other as a fatty acid towards Nile blue. No stain was produced with osmic acid.

Lactic acid among other acids was shown to be absent, and no other organic acid could be detected. The solution obtained by decomposition of the lead salt with H_2S was tested for inulin and other polysaccharides, but without any success.

Thus the lead acetate precipitate consists of the lead salts of malic and succinic acids, with much smaller amounts of the salts of tartaric, phosphoric, sulphuric, and hydrochloric acids.

Sugars.

In general, the method adopted for the estimation of the various sugars is that advocated by Davis and Daish [1913] with certain modifications and additions. Basic lead acetate was added to 200 cc. of the concentrated vine juice until maximum precipitation was effected. The solution was then filtered, the precipitate washed several times with distilled water, and the whole of the filtrate freed from lead by the addition of solid Na_2CO_3 until no more precipitate was produced. The filtrate from the lead carbonate was then made up to 500 cc. with distilled water, and this water-clear solution used for all the sugar-estimations (solution *S*). These estimations were all carried out on the day of preparation of this solution and the fermentation experiments were started on the same day, but were not completed, of course, until about one month later.

Portions of the solution were hydrolysed with citric acid, an invertase solution, and also with dilute HCl, and the reducing power, estimated by Bertrand's method, and the polarimetric value of the solution before and after

hydrolysis determined. The three methods gave results which were in very close agreement with each other and the results showed that with the exception of a small amount of cane-sugar, no disaccharide was present.

The absence of maltose was confirmed by the fermentation experiments suggested by Davis and Daish. A portion of solution *S* was freed absolutely of lead by treatment with H_2S, filtration and the subsequent removal of excess of H_2S by passing a current of air for two hours, and was then diluted with water. Aliquot portions of this solution were autoclaved, inoculated with *S. exiguus* (a maltase-free yeast) and incubated for 28 days. An equal number of controls were set up with ordinary brewer's yeast as the fermenting agent. At the end of the incubation period, the reducing powers of the solutions were determined and the differences between the experiments and their controls found to be extremely small and within the limits of experimental error. Thus the amount of maltose present is negligible. When estimating the reducing power of solution *S*, the hydrolysed solutions, etc., a small correction has been made to allow for the reducing action of the small amount of non-fermentable reducing substances present. An attempt was made to determine the nature of these non-fermentable reducing substances by fermenting more of the solution with *S. exiguus*, precipitating the protein matter with alumina cream, and testing the filtrate for pentoses. This solution on concentration had a slight reducing action towards Fehling's solution, gave a positive phloroglucinol test, but a negative Bial's orcinol reaction, and it is suggested that the substance concerned may be galactose. Some support of this view was obtained by the isolation of a very small amount of a crystalline substance on oxidation with conc. HNO_3, for this crystalline deposit appeared to be identical with mucic acid as far as solubility and crystalline form are concerned.

With regard to the monosaccharides, these appear to consist entirely of glucose and fructose, but the only method of calculating the amounts of each present which can be applied here, is that based on the relationship between the reducing power and the polarimetric value, after allowances have been made for the cane-sugar and non-fermentable substances present.

Preparations of the osazones from solution *S* gave phenylosazones which in crystalline form and in M.P. were identical with glucosazone. Repeated attempts were made to separate other osazones by making use of their different solubilities in hot and cold water, methyl alcohol and ethyl alcohol, but no other osazone crystals could be detected under the microscope. In addition the absence of galactose, and lactose, in any appreciable quantities was confirmed by the inability to obtain more than a trace of mucic acid from solution *S* on oxidation with conc. HNO_3.

Experimental details.

S. Filtrate from the precipitate with basic lead acetate, deleaded and made up to 500 cc.

A. 30 cc. solution *S* diluted to 100 cc. and the reducing sugars estimated by Bertrand's method.

B. 60 cc. solution *S* rendered just acid to methyl orange with conc. H_2SO_4, 4 cc. of a conc. invertase solution added and incubated for 72 hours. The solution made up to 100 cc. and the polarimetric and reducing values determined. A control was set up with 60 cc. H_2O, 4 cc. invertase solution and 2 cc. toluene.

C. 60 cc. solution *S* + conc. H_2SO_4 until just acid to methyl orange and 6 g. citric acid boiled for 10 minutes, neutralised with NaOH and made up to 100 cc. The reducing power and polarimetric value after decolorisation with animal charcoal determined as for solution *B*.

C'. As in *C*, except that HCl was used as the hydrolytic agent instead of citric acid. The results obtained were almost identical with those of *C* and so are not recorded below.

D. H_2S was passed through 40 cc. of solution *S* to delead completely. No precipitate could be observed, but the solution was filtered, excess of H_2S removed by a current of air passed through for 2 hours, and the solution made up to 100 cc. Six samples of 10 cc. each were autoclaved, three of these inoculated with *S. exiguus* and to the others small quantities of brewer's yeast added, and all incubated for 28 days. After the addition of alumina cream, each sample was filtered, diluted to 25 cc. and the reducing power determined. The differences between those fermented by the two yeasts proved to be within the limits of experimental error.

Polarimetric values	Solution *S*	Solution *B*	Solution *C*
Rotation calculated for original solution *S*	0·715°	0·675°	0·670°

Thus on inversion the rotation in 20 cm. tube is lowered by 0·0425° at 16° C.

Percentages of sugars in the concentrated juice.

Cane-sugar	By polarimeter	= 0·060 %
	By reduction	= 0·062
	∴ Average cane-sugar	= 0·061
Hexoses	By reduction	= 2·46

Amount of glucose and fructose by polarimeter.

Rotation of solution *S* due to cane-sugar = 0·032°.
∴ Rotation of solution *S* due to glucose and fructose = 0·683° for a 20 cm. tube.
Glucose and fructose in solution *S* by reduction = 0·984 %.
∴ Solution *S* contains 0·865 % glucose and 0·12 % fructose.
Concentrated vine-juice contains 2·16 % glucose and 0·30 % fructose.

Sugars in the original sap.

0·0288 % glucose, 0·0040 % fructose, and 0·00081 % cane-sugar.
Total amounts in 90,300 cc.: 29·64 g. hexoses (26·03 g. glucose and 3·61 g. fructose) and 0·73 g. cane-sugar.

Nitrate and Nitrite.

No organic nitrogen could be detected either in the conc. vine juice or in the two deposits, with the exception of a small amount of nitrogenous matter which was probably associated with the enzymes, and thus the whole of the nitrogen present appears to be in the form of nitrate and nitrite. The presence of nitrite, observed by Priestley and Armstead [1922], was confirmed by using the starch iodide test and also the specific Griess-Ilosvay reagent (α-napthylamine and sulphanilic acid in acetic acid solutions). The estimation of the amounts of nitrogen present in the form of inorganic salts was carried out according to the method advocated by Strowd [1920] and the results expressed as total inorganic N present. The relative amounts of the two acids concerned is not of much significance in view of the prolonged treatment prior to analysis, and of the fact that alterations in the ratio appear to take place to an extent which cannot be controlled. Indeed samples of ordinary vine juice which had

been stored in coloured bottles in a dark cellar, and to which plenty of toluene had been added, had not the same nitrite-nitrate ratio, and therefore the results given below are only of value as a measure of the total inorganic nitrogen present and as quantitative evidence that both nitrate and nitrite are present.

Nitrate and nitrite. 5 cc. of conc. vine juice were diluted to 250 cc. and subjected to the action of 1 g. of Devarda's alloy in alkaline solution. The ammonia produced was distilled over into standard H_2SO_4 and titrated with NaOH; methyl red was found to be more suitable as the indicator than sodium alizarine sulphonate. A control was set up without the alloy and treated in exactly the same way.

Amounts of $N/28$ NaOH used (average of 2) with alloy = 27·50 cc.
" " " without " = 45·02 cc.
∴ Nitrate and nitrite N in 5 cc. concentrated vine juice = 8·76 mg.

Nitrate. The nitrate N in 5 cc. of conc. juice was determined as above after the decomposition of the nitrite by heating with about 0·5 g. aspartic acid.

$N/28$ NaOH used with alloy 28·62 cc.
without " 44·82 cc.

Thus 5 cc. conc. vine juice contain 8·10 mg. nitrate N and 0·66 mg. nitrite N.

Total inorganic N in 90,300 cc. ordinary vine sap = 2·11 g.

In the conc. juice, 1·95 g. of this N is in the form of nitrate and 0·16 g. as nitrite.

Enzymes.

In testing for enzymes, various solutions were used according to the nature of the enzyme concerned. In certain cases the natural sap could be used, while in others the conc. vine juice was found to be more suitable. Both solutions were also dialysed in a very permeable collodion sac against running water for 4–6 days until no sugar could be detected in the sac contents, and these dialysed solutions used for the tests. Another solution employed was a water solution of the precipitate obtained by adding excess of alcohol to 100 cc. of the dialysed conc. vine juice, and all the tests described below were carried out in duplicate or triplicate, different enzyme solutions being used in each case. Toluene was added to all the solutions. Whenever it was thought necessary, the solutions were buffered and also suitable co-enzyme solutions were added to replace any lost by dialysis, while control tests were always carried out, using the boiled sap or enzyme solution.

Diastase. This enzyme was found to be present and could be detected in the dilute sap, conc. vine juice and the two dialysed solutions.

Invertase. None of the sugar-free solutions had any hydrolysing action on cane-sugar, even in the presence of a boiled invertase solution, added to supply any heat-stable co-enzymes necessary. In confirmation, cane-sugar was added to the dilute sap and the reducing power of the solution

determined at intervals. No alteration in the amount of reducing sugars present occurred.

Maltase. The dialysed solutions were added to maltose solutions buffered at p_H 6·7 and incubated for eight days, together with controls. No alteration in reduction took place during the whole of the period of incubation, and attempts to prepare glucosazone at different times all proved to be unsuccessful. Thus maltase is not present.

Oxidases. All solutions were rich in peroxidase, though no satisfactory proof of the presence of a complete oxidase system could be obtained. With benzidine and α-naphthol the solutions gave only the peroxidase action, but with guaiaconic acid, a strong peroxidase and a very weak complete oxidase reaction after about 6 hrs were obtained. The blueing of guaiaconic acid in the absence of H_2O_2 is probably due to the nitrites present, because this action still persists to some extent when the solution is boiled, while the peroxidase action is destroyed by heat. The dialysed solutions and the solution of the precipitate with alcohol gave only the peroxidase action.

Catalase. The ordinary sap was found to contain an exceedingly small amount, and the conc. vine juice a larger amount of the enzyme catalase.

Lipase. This enzyme was tested for qualitatively and quantitatively by using as substrate a suspension of olive oil, and in other cases milk. In no case did any fat hydrolysis take place.

Glycerophosphatase. The method used in the tests for this enzyme is essentially that used by Plimmer [1913]. 400 cc. of ordinary vine juice were added to 100 cc. of 5 % sod. glycerophosphate solution, toluene added, and this solution, together with a control in which boiled vine juice was used, incubated for three weeks. The inorganic phosphate content of each solution was estimated at intervals by the uranium acetate method and also by that used by Plimmer, the gravimetric method. The figures obtained were constant throughout the experiment, indicating that no glycerophosphatase was present.

Inorganic phosphorus present (as P_2O_5) in the whole of the solution.

	Solution A 0·056 g.	0·058	0·052
	Solution B (control) 0·056	0·055	0·054
Total phosphorus (as P_2O_5) = 1·79 g.	Time (days) 0	5	21

The experiment was repeated later with conc. vine juice with the same result.

Proteases. Ordinary vine sap and the conc. juice were incubated for 7–14 days with small amounts of fibrin, and suitable controls were set up. No digestion was apparent and the solutions and controls gave identical amino-acid titrations (formaldehyde method) after seven days. Similar tests were carried out, using Witte's peptone and testing for free tryptophan, with negative results. Thus proteases of similar nature to pepsin and erepsin appear to be absent.

Of the enzymes for which tests were made, diastase, peroxidase, and a trace of catalase were present in the sap, and maltase, invertase, lipase, proteases, glycerophosphatase and rennin were absent.

General summary of the quantitative results.

Twelve vines yielded about 110 litres of sap, of which 90,300 cc. were used for analysis. The figures given below represent the total amounts present in the various precipitates and solutions.

	Total g.	Amount in g. per litre of natural sap
Ash	50·3	0·56
Total solid	140·7	1·56
P_2O_5	2·92	0·032
Reducing sugars	29·64	0·33
Glucose	26·03	0·29
Fructose	3·61	0·04
Cane-sugar	0·73	0·008
Inorganic N	2·11	0·023
Organic acids (oxalic, tartaric, malic and succinic) approx.	50	0·56

In conclusion, I should like to express my indebtedness to Professor Priestley for having supplied the sap, and my heartiest thanks to Professors Priestley and Raper for much helpful advice given to me during this investigation.

Summary and Conclusions.

1. The sap obtained from the bleeding vine is a dilute solution of inorganic and organic substances, containing 1·56 g. of solid matter per litre, of which approximately one-third is inorganic and the remainder organic. These figures are the average figures for a large volume of the sap collected over an extended period of bleeding.

2. The organic constituents are chiefly sugars and organic acids. The sugars present are glucose, fructose, and a very small amount of cane-sugar.

3. The organic acids present are oxalic, tartaric, malic and succinic acids, and the total quantity of these organic acids exceeds that of the sugars present. A small amount of an acid M.P. 161–2° C. was isolated, but could not be identified.

4. The mineral salts present are the chlorides, sulphates, nitrites, nitrates, silicates and phosphates of sodium, potassium, calcium, iron, magnesium, and to a lesser extent manganese and aluminium. The iron present is readily diffusible.

5. Only a very small amount of organic nitrogen is present and this may possibly be attributed to the nitrogenous portion of the enzymes. Thus the whole of the nitrogen appears to be in the form of nitrates and nitrites, though the possibility of amino-acids being present, and of their decomposition with the nitrous acid must not be overlooked.

6. No lipins could be detected in the concentrated sap. A mere trace of a fat-like substance was isolated, but in quantities which did not allow of any tests other than those of solubility and staining. The latter tests suggest that the substance isolated was a mixture of neutral fat and free fatty acid.

7. The enzymes diastase, peroxidase, and to a small extent catalase, are present in the sap, while maltase, invertase, lipase, proteases, glycerophosphatase and rennin were found to be absent.

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CLV. STUDIES ON KETOSIS: I. THE RELATION BETWEEN ALKALOSIS AND KETOSIS.

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The appearance of acetone bodies in the urine (ketosis) occurs in man in many diverse conditions, and is usually associated with an upset in the metabolism of carbohydrate or with damage to the liver function. Davies, Haldane and Kennaway [1920] found that ketone bodies appear in small concentrations in the urine during experimental alkalosis; and it has recently been shown by Haldane, Wigglesworth and Woodrow [1924] that concurrently with this acetonuria there is a considerable disturbance of the normal metabolism of carbohydrate. The main object of the present work was to determine the relation between the ketosis of carbohydrate inanition and the acetonuria induced by alkalosis.

Methods.

The experiments have been carried out on rats. Rats of all ages have been employed at different times, but where accurate comparisons have been required, rats of about 100 g. selected from the same litter and reared under the same conditions have been used. The small rats have, as a rule, been used in pairs. Even under these conditions individual variations are considerable.

The urine of the rats has been obtained free from faeces in the usual way and collected over twenty-four hour periods in a small conical flask containing a few cc. of water covered with a layer of toluene. It was made up to 100 cc. with the washings of the collecting funnel and estimations made upon aliquot portions containing up to 15 mg. of ketone substances.

Acetone and acetoacetic acid (estimated together throughout as "total acetone") and β-hydroxybutyric acid have been determined on the same sample of urine by a method based on the Messinger iodometric titration and the Shaffer oxidation of β-hydroxybutyric acid with potassium dichromate.

The urine is placed in a 500 cc. round bottom flask, 25 g. of anhydrous sodium sulphate and 1 cc. of concentrated sulphuric acid are added and the volume made up to about 300 cc. with distilled water. The flask is fitted with a dropping funnel and a delivery tube leading to the bottom of another 500 cc. flask containing 100 cc. of 4 % caustic soda. The delivery tube from this

flask leads to a condenser fitted with an adapter which dips below the surface of the fluid in the receiver.

The liquid in both flasks is heated to boiling and distillation continued for ten minutes into a receiver containing 5 cc. of 25 % caustic soda and a known volume (excess) of 0·01*N* iodine. 5 cc. of 25 % sulphuric acid are then added to the distillate and the excess iodine titrated with 0·01*N* sodium thiosulphate. 1 cc. of 0·01*N* iodine = 0·0967 mg. acetone. A blank determination is made on the reagents and this value deducted.

At the end of the ten minutes the first receiver is replaced by a second receiver containing 5 cc. of 40 % soda. The gas is lowered so that the liquid only just boils and 25 cc. of the solution of potassium dichromate[1] allowed to run in drop by drop during 30 minutes. The solution is then boiled rapidly for ten minutes. 0·5 cc. of 30 % hydrogen peroxide (Merck's Perhydrol) is added to the distillate, which is transferred to a 500 cc. flask, brought cautiously to the boil and distilled for ten minutes, the acetone being estimated exactly as before. A blank determination is again made on the reagents.

As in the original method of Shaffer there is a constant yield of acetone from β-hydroxybutyric acid of about 85 % of the theoretical. The cc. of 0·01*N* iodine are therefore multiplied by the factor 0·2 to give milligrams of β-hydroxybutyric acid.

Lactic acid has been estimated in urine by the method of Clausen [1922]. The ether extraction method of Clausen has been employed, the phenols being first removed by extracting with ether at p_H 7·0. The error due to β-hydroxybutyric acid has been eliminated, as he suggests, by the method of Shaffer, the distillate being redistilled after the addition of 0·5 cc. of 30 % hydrogen peroxide and 5 cc. of 40 % soda. In the experiments in which lactic acid determinations have been made, the urine has been collected in dry vessels, and a minimal quantity of water used in washing out the vessels and making up to a known volume.

The following are the diets used in the experiments reported:

Diet 1, a normal diet for experiments of short duration: casein 10, starch 50, sugar 17, lard 15, salts 2.

Diet 2, a diet free from carbohydrate but containing some protein: casein 10, lard 50, salts 2.

Diet 3, free from both protein and carbohydrate: filtered butter 100, salts 3.

The salts employed had the following composition: normal calcium phosphate 15, di-sodium hydrogen phosphate 10, potassium di-hydrogen phosphate 27·5, sodium chloride 10, potassium chloride 5.

Ketosis occurring in rats fed upon a diet deficient in carbohydrate.

When man is deprived of carbohydrate ketosis rapidly sets in; but this is not commonly the case with other animals [Baer, 1906]. The rat does not appear to have been studied in this connection.

[1] 20 g. potassium dichromate, 250 cc. concentrated sulphuric acid, water to 1 litre.

The urine of rats, particularly old individuals, gives a positive reaction to the nitro-prusside test, which is due at least in part to acetone, since the distillate from such urine also gives a positive test. The normal excretion of "total acetone" ranges from 0·0–0·30 mg. per diem, and of β-hydroxybutyric acid from 0·8–2·5 mg.; though, as will be shown later, the latter is subject to wide variations.

Table I represents a typical experiment showing the response of the rat to a diet of fat alone (diet 3).

Table I.

Figures in milligrams per diem.
Two young rats fed on diet 3 after 3. vi. 24.

Date	Weight g.	"Total acetone"	β-hydroxybutyric acid
3. vi. 24	133	0·00	0·70
	107		
4	124	0·06	1·42
	101		
5	122	1·45	4·56
	98		
6	120	6·34	21·20
	96		
7	120	1·86	5·08
	97		
9	121	0·31	1·44
	96		

It is clear that the rat shows a very definite ketosis on this diet. The excretion of "total acetone" and of β-hydroxybutyric acid rises for the first few days of the experiment reaching its maximum at the third or fourth day. At the height of ketosis the output of β-hydroxybutyric acid is usually between two and four times that of the "total acetone." In a few cases a secondary rise in ketosis has occurred at about the tenth day, but when this has been the case the excretion has soon returned to normal once more. The rats show a rapid fall in weight during the first two days but thereafter the loss in weight usually does not exceed 1 g. per diem and may often remain constant for several days during the experiment. The height to which the ketosis may rise is exceedingly variable, but as in the case of man it is much more marked in young animals, and in a few old individuals has been almost absent.

Rats fed on diet 2 (*i.e.* with some protein added to the fat) ordinarily show a much less intense ketosis, though the time relations of the adaptation are the same as before. Such an experiment is given in Table II.

Table II.

Figures in milligrams per diem.
Rat (about 200 g.) fed on diet 2 after 3. iii. 24.

Date	"Total acetone"	β-hydroxybutyric acid
3. iii. 24	0·13	1·95
4	0·19	1·31
5	0·83	1·92
6	1·76	3·18
7	1·31	3·50
8	0·41	1·78
9	0·29	1·22

If the animal is killed after about ten days on a fat diet, when the ketone excretion is within normal limits, the liver is seen to be small in size and laden with fat. Otherwise the organs appear perfectly normal.

It has long been known that the body possesses a considerable power of adaptation to a diet rich in fat. It is clear that in the rat this adjustment occurs with remarkable rapidity, even when the only antiketogenic element in the diet is the glycerol of the fat itself. Moreover it is worth noting in Table I that after the first three days on the fat diet there was practically no further loss of weight during the experiment. That is to say, at the time when the rat is showing the rapid adjustment to the fat diet (as judged by the ketosis) there is no evidence that it is drawing upon any endogenous source of antiketogenic substance. It is difficult to reconcile these results with the Shaffer [1921] theory of antiketogenesis.

The effect of alkalosis on the excretion of ketone bodies by the rat.

It has long been known that the administration of sodium bicarbonate to diabetics causes a marked increase in the excretion of ketone bodies. Similarly in experimental alkalosis in the normal subject a slight ketosis may occur. Most authors, in discussing the ketonuria of these conditions, have described the appearance of the organic acids in the urine as a regulatory mechanism for neutralising the alkali. Haldane, Wigglesworth and Woodrow [1924], however, obtained evidence that simultaneously with the ketosis there was a considerable upset in the metabolism of carbohydrate; and they were inclined to attribute the ketosis to a more profound disturbance of metabolism than would be implied by a mere "washing out" of these substances by an excess of alkali. The experiments here described were made upon the rat in an attempt to throw further light on this point.

In Table III is shown a typical experiment in which 1 g. of sodium bicarbonate per diem was added to the normal diet.

Table III.

Figures in milligrams per diem.
Rat of about 200 g.

Date	"Total acetone"	β-hydroxybutyric acid	Remarks
6. ii. 24	0·25	0·86	Diet I
7	0·28	1·3	„ +1 g. $NaHCO_3$
8	0·16	8·87	„ „
9	0·28	10·32	„
10	0·19	4·59	

Under these conditions there is a very marked rise in the excretion of β-hydroxybutyric acid but the output of acetoacetic acid is unaffected. In experiments in which the administration of bicarbonate has been continued for many days, there has been no sign of any drop in the β-hydroxybutyric acid excretion indicating an adjustment to the condition. If the animal be

killed after the β-hydroxybutyric acid excretion has been at a high level for several days and its liver examined, this shows no fatty infiltration as it does during carbohydrate inanition.

In an investigation of the mechanisms employed by the body in combating an excess of alkali, McLeod and Knapp [1919] found that administration of sodium carbonate caused an increase in the excretion of lactic acid. Since the method (the v. Fürth-Charnass permanganate oxidation) employed by these workers would also estimate considerable quantities of β-hydroxybutyric acid, it seemed desirable to repeat the experiments making simultaneous estimations of the acetone bodies and the lactic acid. Table IV illustrates such an experiment.

Table IV.

Two rats (each about 150 g.). The output of acids in milligrams per diem.

Date	"Total acetone"	β-hydroxybutyric acid	Lactic acid	Remarks
2. vi. 24	0·19	2·20	4·1	Diet 1
3	0·36	2·25	3·27	„ +2 g. $NaHCO_3$
4	0·34	15·30	10·4	„ „
5	0·20	23·7	11·6	„ +2 g. NH_4Cl
6	0·19	3·78	3·90	

It will be seen that whereas again the total acetone excretion is not affected, the β-hydroxybutyric acid and lactic acid outputs rise. In this experiment the former is more affected than the latter, but this is not constantly the case. On the 5th, 1 g. of ammonium chloride was added to the normal diet bringing about a rapid reversal of the bicarbonate effect.

As in the experiments of McLeod and Knapp the part which these acids can play in neutralising the excess of alkali is quantitatively very small. For example on 5. vi. in Table IV, the titratable alkalinity of the urine was 89·5 cc. *N*/10 alkali, whereas the β-hydroxybutyric and lactic acids together will account only for about 3·4 cc. *N*/10 alkali. Moreover, the extent to which they rise is by no means directly proportional to the alkalinity of the urine. In fact any substance which during its metabolism will liberate an excess of base will apparently bring about this increased excretion of organic acids, while the urine may be only very slightly alkaline or even slightly acid.

For example, it was observed in the case of a rat fed on a diet containing calcium lactate in the salt mixture that the excretion of β-hydroxybutyric acid rose from 2·3 mg. per diem to 9·5 mg. with the urine neutral or slightly acid. After administration of ammonium chloride it fell to 0·90, and rose again to 5·64 mg. when this was stopped. It is noteworthy in this experiment that administration of acid reduced the β-hydroxybutyric acid excretion to considerably *below* the normal level of about 2 mg. for a rat of this size.

Similar results have been obtained after administration of sodium acetate. In one case the excretion of β-hydroxybutyric acid rose from 2·1 mg. to 23·92 mg. after sodium acetate had been added to the food for one day. Doubtless many organic acids would behave in the same way; hence it follows that experiments based on the increase in the excretion of lactic, β-hydroxy-

butyric and possibly other acids after the administration of the sodium salts of other substances must be interpreted with caution.

It was of interest to see whether "alkali-forming foods" would have the same effect upon the output of organic acids as feeding with alkalies. With this object rats were fed on diet 1 with the addition of carrots. Table V gives the results of such an experiment.

Table V.

Output of acids in milligrams per diem.
Two rats (each about 160 g.).

Date	"Total acetone"	β-hydroxybutyric acid	Lactic acid	Remarks
20. vi. 24	0·10	1·4	3·1	Diet 1
21	0·14	1·35	2·9	„ + carrots
22	—	—	—	„ „
23	0·17	5·55	13·4	„
24	0·23	4·8	13·5	„
25	0·22	4·03	4·74	„
26	0·23	1·62	2·5	„

The excretion of organic acids is seen to rise as after the administration of sodium bicarbonate. It is noteworthy that the output had not returned to normal until three days after the carrots had been discontinued. This may perhaps be due to the gradual liberation of base in the tissues during metabolism. The effect of sodium bicarbonate administered as such seldom persists for more than one day. A similar "lag" may sometimes be seen in the onset of the increased excretion of organic acids, as compared with the changes in titratable acidity, after the administration of sodium bicarbonate, and suggests that the changes in their production occur in the tissues and not during the secretion of urine.

The effect of glucose on the excretion of organic acids after administration of alkali.

Since carbohydrate has such a powerful influence in depressing the excretion of β-hydroxybutyric acid in ketosis it was interesting to see whether it had any influence on the excretion of this acid in alkalosis. Though the rats were on a diet rich in carbohydrate it seemed possible that massive doses of glucose might affect equilibria in the body in such a way that the excretion of the acids might be affected. Tables VI, VII, and VIII, show experiments on these lines.

Table VI.

Rat of about 200 g. weight on diet 1.

Date	"Total acetone"	β-hydroxybutyric acid	Remarks
25. iii. 24	—	—	5 cc. of 10 % $NaHCO_3$ injected at 12.15 p.m.
26	0·14	10·79	$NaHCO_3$ as 25th at 12.15 + 5 cc. of 20 % glucose
27	0·14	5·18	$NaHCO_3$ at 12.15 as before
28	0·14	9·25	

Table VII.

Rat (about 200 g.) on diet 1 with addition of 2 % calcium lactate.

Date	"Total acetone"	β-hydroxybutyric acid	Remarks
26. v. 24	0·16	8·86	5 cc. of 20 % glucose injected
27	0·23	6·56	
28	0·23	11·44	

Table VIII.

Two rats (each about 160 g.) fed with diet 1 with the addition of 2 % sodium bicarbonate after 21. vi. 24.

Date	"Total acetone"	β-hydroxybutyric acid	Lactic acid	Remarks
21. vi. 24	0·28	2·25	3·37	
23	0·00	44·5	10·4	5 cc. of 20 % glucose injected to each
24	0·00	37·0	17·2	
25	0·20	49·6	8·6	Glucose given as on 23rd
26	0·00	39·0	15·1	
27	0·19	52·2	11·9	

In Table VI a marked depression of the β-hydroxybutyric acid excretion due to sodium bicarbonate is seen to occur after injection of 5 cc. of 20 % glucose. Table VII illustrates a similar effect when the high β-hydroxybutyric acid output is due to the calcium lactate in the diet. Table VIII shows an experiment in which simultaneous estimations of lactic acid were made. While the β-hydroxybutyric acid excretion shows the usual depression, the lactic acid output seems to be increased. The mode of action of the glucose in these experiments is not clear. It does not appear to be due to its evoking an increased secretion of "insulin" by the pancreas, since injections of insulin have not this effect.

A comparison of the effects of alkalosis with the ketosis due to a diet of fat.

The experiments described have shown that the administration of alkalies causes in the rat a rise in the excretion of β-hydroxybutyric acid and lactic acid, but little or no change in the excretion of acetoacetic acid; whereas during fat feeding both acetoacetic and β-hydroxybutyric acids increase in the urine. Table IX shows an experiment in which the two procedures were carried out successively on the same animal. There are the usual changes during the bicarbonate alkalosis: a rise in the excretion of β-hydroxybutyric and lactic acids. The rat was an old animal so that the ketosis on the fat diet was not very intense, but there was a definite rise in the acetoacetic acid and β-hydroxybutyric acid excretion while the output of lactic acid tended to fall. Bicarbonate was administered after adaptation to the fat diet was complete, at first in the food and then by injection. The β-hydroxybutyric acid rose greatly while the lactic acid, though definitely affected, was apparently less readily so than when the rat was on a mixed diet. The acetoacetic acid showed a slight rise. This point will be returned to later.

Table IX.

Figures in milligrams per diem.
Rat (about 300 g.) previous to experiment fed on diet 1.

Date	"Total acetone"	β-hydroxybutyric acid	Lactic acid	Remarks
5. vi. 24	0·22	2·21	3·1	Diet 1 + 2 % $NaHCO_3$
6	—	—	—	" "
7	0·08	20·50	19·0	" + 2 % NH_4Cl
8	—	—	—	" "
9	0·08	3·06	2·87	Diet 3
10	0·10	2·40	2·7	"
12	3·92	10·95	2·63	"
13	2·12	3·99	2·16	"
15	0·54	1·23	1·77	"
16	—	—	—	" + 2 % $NaHCO_3$
17	0·35	19·0	2·63	" + 5 cc. 10 % $NaHCO_3$ injected
18	2·1	30·6	8·0	

The differences between the two types of experiment are very clearly shown in Fig. 1.

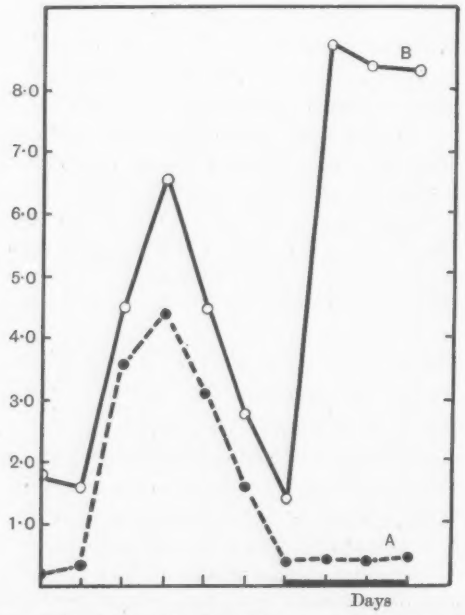

Fig. 1. Abscissa: days. Ordinate: excretion in milligrams per diem. Curve A, "total acetone." Curve B, β-hydroxybutyric acid. Rat of about 250 g. weight, fed throughout on diet 3. The thickened portion of the base line indicates the days on which 5 cc. of 10 % sodium bicarbonate were injected.

The experiments so far described seemed to indicate that there is a very definite distinction between the "ketosis" due to alkalosis in the rat and the true ketosis of carbohydrate inanition. In the experiments of Haldane, Wigglesworth and Woodrow [1924], however, the occurrence of the ketosis synchronising with the upset in carbohydrate metabolism and with a very

low respiratory quotient, suggests very strongly that we have here to do with a real disturbance of fat metabolism—brought about either directly or indirectly as the result of a deranged carbohydrate metabolism. In this same work it was noted that the excretion of β-hydroxybutyric acid was much more affected than that of acetoacetic acid and acetone: analogous, that is, to the condition described in the rat. It was thought that the distinction might lie in the fact of the rat being more resistant to this treatment with alkalies than is man; and that more massive doses of sodium bicarbonate might reproduce this condition in the rat. Table X shows an experiment of this nature which has been confirmed several times.

Table X.

Rat of about 180 g.

Date	"Total acetone"	β-hydroxybutyric acid	Remarks
11. ii. 24	0·20	1·30	Diet 1 + 4 % $NaHCO_3$
12	0·16	8·87	„ + 5 cc. of 10 % $NaHCO_3$ injected
13	0·28	16·7	„ + 10 cc. of 10 % $NaHCO_3$ injected
14	1·13	20·3	„
15	0·82	5·15	„

It will be seen that when the dose of sodium bicarbonate was increased to 10 cc. of 10 % solution, an undoubted rise in the total acetone excretion occurred, and this persisted until the next day.

These results do not necessarily prove that the normal metabolism of fat has been interfered with; but taken in conjunction with experiments to be described later on the effect of alkalosis during adaptation to a fat diet, they do suggest that in certain individuals, or when the intensity of the alkalosis is sufficient, some essential step in fat metabolism is obstructed. In any case it is not easy to picture the intimate differences between the two conditions. Perhaps in mild alkalosis we have to do with a direct effect of alkali on chemical equilibria in the body whereas when the alkalosis is intense we may be affecting factors which control the normal oxidation of fats. If this point of view is correct, then during alkalosis in man we encounter a twofold phenomenon: an excretion of organic acids analogous to the effect of mild alkalosis in the rat and, superimposed on this, a slight true ketosis.

The influence of sodium bicarbonate on adaptation to a diet of fat.

Since it appears that large doses of alkali may upset the metabolism of fats, it was of interest to see whether the adaptation to a diet of fat would be interfered with if bicarbonate were added to the food throughout the experiment.

A few experiments bearing on this point have already been mentioned. In that detailed in Fig. 1, administration of sodium bicarbonate after the adaptation was complete had no effect on the "acetone" excretion, but caused a great rise in the β-hydroxybutyric acid output. In Table IX the acetoacetic

acid excretion showed a tendency to rise also (though to a less extent), and in other rats it has behaved similarly. It seemed probable that variations in the intensity of the alkalosis and in the sensitivity of different rats were the causes of these differences. The question was now investigated in greater detail.

Table XI shows the first experiment of this series. The rats (B) with 6 % of sodium bicarbonate in the fat showed an immediate rise in the β-hydroxybutyric acid excretion, but the output of total acetone ran approximately

Table XI.

Four rats from the same litter (about 120 g. each).

	A. Fed on diet 3 after 12. v. 24		B. Fed on diet 3 with the addition of 6 % $NaHCO_3$	
Date	"Total acetone"	β-hydroxybutyric acid	"Total acetone"	β-hydroxybutyric acid
12. v. 24	0·06	2·25	0·07	2·0
13	0·53	2·0	0·10	20·25
14	6·3	15·16	8·4	53·9
15	19·2	48·5	14·2	86·0
16	4·3	12·44	1·5	36·9
17	—	—	0·55	28·3
18	1·37	3·86	0·82	25·5

parallel with that of the controls. After the third day there was a perfectly definite return of the acetoacetic acid excretion to normal, the β-hydroxybutyric acid remaining high in the bicarbonate rats.

From this experiment it appeared as though the two forms of organic acid excretion were simply behaving additively—the alkali having no influence on the course of the ketosis. This, however, has not been the invariable result. In fact in all of the eight cases in which precisely the same experiment has been repeated, both the intensity of the ketosis and the occurrence of adaptation have been definitely affected. Evidently we have here once more to do with differences in the sensitivity of the rats.

In all the latter experiments, though the ketosis has occasionally shown a slight subsidence towards the tenth day, the rapid adjustment invariably shown by the controls has been completely eliminated. Usually the ratio of β-hydroxybutyric acid to total acetone output has been higher in the bicarbonate experiments. The experiment detailed in Table XII is quite characteristic and will serve to illustrate the main points.

In the bicarbonate rat (C) the ketosis developed more rapidly than in the controls, rose to a higher maximum, and beyond a slight subsidence after the third day, showed no tendency to return to normal by the eighth day; whereas the controls were almost completely adapted by the fifth day.

In order to see whether the power of adaptation had been permanently affected the bicarbonate was discontinued in the diet of (C) on the 16th. It will be seen that though the bicarbonate had been got rid of by the next day (as evidenced by the acidity of the urine), the ketosis subsided only gradually.

On the 15th, 6 % sodium bicarbonate was added to the diet of (B); *i.e.* these rats were given the same diet as (C) had been having. The β-hydroxybutyric acid excretion rose, and so to a less extent did the total acetone; but the effect was very much less than that shown by the rats (C) which had been on this diet throughout. On the other hand the addition of double this quantity of sodium bicarbonate to the diet of (A) at the same time brought about a complete collapse of the adaptation which had been established. This is typical of the results obtained on other rats. Up to a certain dose of bicarbonate the β-hydroxybutyric acid alone is affected; but when the alkalosis reaches a certain intensity the adaptation seems to break down and both acetoacetic and β-hydroxybutyric acids rise to very high concentrations in the urine.

Table XII.

Six rats from the same litter (about 80 g.).

	A. Fed on diet 3 after 8. vii. 24		B. Fed as for A		C. Fed on diet 3 with 6 % $NaHCO_3$	
Date	"Total Acetone"	β-hydroxybutyric acid	"Total Acetone"	β-hydroxybutyric acid	"Total Acetone"	β-hydroxybutyric acid
8. vii. 24	0·08	1·78	0·05	1·46	0·06	* 1·44
9	0·47	2·76	0·33	1·64	0·93	5·04
10	12·67	31·7	7·3	16·0	38·2	120·1
11	16·33	46·0	12·56	36·6	46·0	126·0
12	28·5	81·4	34·8	92·0	35·5	102·2
13	1·69	4·5	6·1	18·8	34·3	98·5
14	1·74	4·10	0·34	2·0	42·7	103·2
15	0·72	2·8 *	0·48	1·82†	30·3	97·6
16	13·82	46·2	0·72	3·2	32·6	101·4‡
17	43·2	131·0	2·5	13·0	28·6	73·6§
18	—	—	4·4	20·0	12·57	30·0

* 12 % $NaHCO_3$ added. † 6 % $NaHCO_3$ added. ‡ $NaHCO_3$ stopped. § Urine acid.

These experiments fully bear out the views put forward above: that when the alkalosis exceeds a critical intensity the ketosis is accentuated. They further suggest that the ketosis is more susceptible to the effect of alkali during the stage of adaptation than after this has been established. Moreover, the adjustment of young rats seems to be more easily broken down than does that of older animals, a fact which is probably correlated with the greater susceptibility of the former to ketosis.

The effect of acids on the ketosis due to a diet of fat.

The effect of acids in causing a depression of the ketonuria of diabetes has never been so definite as the converse effect of alkali. Recently, however, Adlersberg [1923] has shown that there is a marked diminution of ketosis in man, in diabetes and during carbohydrate inanition, after giving ammonium di-hydrogen phosphate.

It has been seen that ammonium di-hydrogen phosphate or ammonium chloride given to a rat on a normal diet leads to a depression in the excretion

of organic acids. These salts were now administered along with a diet of fat alone, to see what effect they would have on the adaptation to this diet.

When rats were fed on diet 3 with the addition of 3 % of ammonium dihydrogen phosphate, variable results were obtained. In some cases the intensity of the ketosis was considerably depressed but in others it was scarcely affected. When however the content of the phosphate was increased up to 10 %, a very marked suppression of the ketosis occurred, and in some cases this was almost completely eliminated. This effect is probably not due to the phosphate ion since addition of a neutral mixture of phosphates did not affect the ketosis. Moreover, the same change is shown when the ammonium phosphate is replaced by ammonium chloride, which would tend rather to rob the body of phosphate. Two experiments showing an average degree of suppression of the ketosis, one with ammonium phosphate and one with ammonium chloride, are given in Tables XIII and XIV. The former salt is usually the more effective. The time relations of the rise and fall in the ketosis do not seem to be constantly affected.

It would appear then that acids have an effect on ketosis just the converse to that of alkalies, and that they exert a favourable influence on the metabolism of fats.

Table XIII.

Four rats from the same litter about 65 g.

	A. Diet 3 after 19. vii. 24		B. Diet 3 + 10 % $NH_4H_2PO_4$	
Date	"Total acetone"	β-hydroxybutyric acid	"Total acetone"	β-hydroxybutyric acid
19. vii. 24	0·00	0·75	0·04	0·69
20	0·62	2·68	0·04	0·88
21	32·0	100·8	3·58	8·4
22	28·4	100·6	5·17	10·1
23	10·3	22·9	1·11	2·7
24	3·82	8·2	0·48	1·6

Table XIV.

Four rats from same litter (each about 60 g.).

	A. Diet 3 after 21. vii. 24		B. Diet 3 + 8 % NH_4Cl	
Date	"Total acetone"	β-hydroxybutyric acid	"Total acetone"	β-hydroxybutyric acid
21. vii. 24	0·00	0·88	0·06	0·76
22	0·27	1·6	0·15	0·80
23	25·3	65·2	16·4	35·3
24	17·6	42·4	6·0	14·8
25	13·7	30·6	1·5	4·2
26	5·46	12·2	0·29	2·2
27	1·35	3·8	0·24	1·8

A note on alkali therapy.

One practical point deserves mention. It has long been customary to treat the depletion of the alkali reserve of the diabetic by administration of sodium bicarbonate. So long as the increased excretion of acetone bodies which such

treatment evokes was regarded as a mere "washing out" of these substances, there was no evidence that the alkali was actually harmful to metabolism. Haldane, Wigglesworth and Woodrow pointed out that the disturbances in carbohydrate metabolism induced by large doses of bicarbonate were in many respects so like the metabolic disturbances of diabetes as to suggest that the alkali might well accentuate the disease. The present researches show that so far as concerns the metabolism of fat in the absence of carbohydrate—which is the problem always before the diabetic—large amounts of alkali have a definitely inhibitory influence.

Summary.

A modification of existing methods for the estimation of acetone and β-hydroxybutyric acid in small amounts of urine is described.

The normal urine of the rat contains from 0·0–0·30 mg. of "total acetone," and from 0·8–2·5 mg. of β-hydroxybutyric acid per diem.

When deprived of carbohydrate in the diet (and particularly upon a diet of fat alone) the rat shows a marked ketosis. This ketosis increases during the first few days, reaches its maximum at about the third day, and thereafter subsides rapidly, adaptation being usually complete by the fifth day. The ketosis is much more marked in young animals. At the height of the ketosis the ratio of β-hydroxybutyric acid to total acetone is usually between 2 and 4.

Administration of sodium bicarbonate to the rat causes a rise in the output of lactic and β-hydroxybutyric acids but no change in the excretion of total acetone. Acids reverse this effect. There does not appear to be any adaptation to this condition.

Calcium lactate, sodium acetate (and probably the alkali salts of other oxidisable organic acids) have the same effect as alkali. Alkali-forming food (carrots) has the same effect. The bearing of these facts on the results of feeding experiments is pointed out.

The injection of large doses of glucose during the alkalosis causes a fall in the excretion of β-hydroxybutyric acid.

If very large amounts of sodium bicarbonate are injected into the rat the total acetone excretion shows a distinct rise. This is interpreted as indicating a real disturbance in the metabolism of fat.

With one exception the presence of 6 % of sodium bicarbonate in a diet of fat has eliminated the usual adaptation. The adaptation occurs when the alkali is discontinued. The adaptation seems to be less susceptible to alkali once it is established; but sufficiently large doses of sodium bicarbonate will lead to its being broken down completely.

Ammonium di-hydrogen phosphate and ammonium chloride will, in sufficient concentration, cause a suppression of the ketosis due to a diet of fat.

The bearing of these findings upon alkali therapy is pointed out.

I wish to express my most sincere thanks to Prof. F. G. Hopkins for his help and encouragement throughout this work. I wish also to thank Prof. Hopkins for superintending the feeding experiments and Mr C. E. Woodrow for carrying out the necessary injections. My acknowledgements are due to the Scientific and Industrial Research Board for a grant held during this research.

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CLVI. STUDIES ON KETOSIS: II. THE OXIDATION OF KETONE BODIES BY THE ISOLATED LIVER OF THE RAT.

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It has been generally assumed that the liver is primarily concerned in the metabolism of the ketone bodies. It was thought that an investigation of the oxidation of these substances by liver preparations—using the oxygen uptake as a measure of oxidation—might throw further light on the changes occurring in the body during the adaptation to a fat diet and possibly on the part played by antiketogenic factors in the metabolism of these substances.

Methods.

The oxygen uptake has been measured upon 0·5 g. quantities of fresh liver preparations from the rat by means of the modified Barcroft differential blood-gas analysis apparatus as employed in this laboratory. The reaction bottles were calibrated to take 4 cc. of fluid; and four apparatus were used at the same time in one bath at 37°.

Various methods of preparing the liver so as to give good agreement between the several samples have been tried. The method finally adopted was the following. The rat was anaesthetised with chloroform and bled as thoroughly as possible. About 3 g. of liver were removed under conditions as aseptic as possible, placed upon a glass plate and covered with a small metal capsule sealed round the edges with plasticine. The whole was immersed in a freezing mixture of ice and salt until the tissue was just frozen through (15 minutes). The capsule was then removed and the liver cut by hand into slices as uniform as possible of rather less than 0·5 mm. thickness. Consecutive slices were transferred to four watch glasses so as to give the most uniform distribution possible until these contained 0·5 g. of tissue. With these precautions excellent agreement could be obtained between four samples from a given liver.

The tissue was then transferred to the reaction bottles and 4 cc. of buffer solution added to each. The buffer employed was either phosphate mixture

of p_H 7·4 or Locke-Ringer solution to which a knife-point of solid calcium carbonate was added. When the usual amounts of tissue were present the filtrate from the latter mixture had a p_H of about 7·4. It is of interest to note in passing that no difference was to be observed between the rate of oxygen uptake by the tissue in phosphate buffer and the tissue in Ringer. Hopkins has recently found that the oxidations catalysed by glutathione are markedly inhibited in phosphate buffer.

All the substances whose oxidation has been studied have been added so as to give a concentration of 1/25th molecular in the presence of the tissue, the reaction of the solutions being adjusted to p_H 7·4 by addition of sodium hydroxide or hydrochloric acid.

Acetoacetic acid has been prepared by the method of Hurtley as employed by Shaffer [1921]. Inactive β-hydroxybutyric acid neutralised with sodium hydroxide has been employed. These two substances have been adjusted to the same molecular concentration by means of estimations by the method described in the preceding paper.

Mention may here be made of a possible source of error. If the sodium hydroxide used in neutralisation contains much carbonate and if sufficient time be not allowed for equilibration in the water-bath, the subsequent absorption of the carbon dioxide liberated on warming will give a false appearance of increased oxygen uptake.

Results.

Under these conditions the liver of the rat shows an increased uptake of oxygen in the presence of β-hydroxybutyric acid and of acetoacetic acid. The intensity of this oxidation was rather variable and seemed to be particularly marked in large old rats. Fig. 1 shows a typical experiment. It will be seen that the acetoacetic acid is more readily oxidised than the β-hydroxybutyric acid; though even in the case of the former the amount of oxygen taken up does not exceed 1/5th of an atom of oxygen per molecule of acid in five hours.

Fig. 2 shows an experiment upon the liver of a similar rat after 36 hours' fast, *i.e.* a liver which must have been almost entirely free from glycogen. There is no apparent change in the oxidation of the ketone bodies.

Fig. 3 represents a similar experiment after the rat had been for three days on a diet of butter fat alone; *i.e.* at a time when the ketosis was probably most intense. There is again no definite change in the oxidation.

Fig. 4 is from a similar rat after keeping for ten days on a diet of butter fat; *i.e.* after the rat had become adapted to the fat diet. The liver at this stage was very small and laden with fat, so that the results are not strictly comparable with the preceding. The total oxygen uptake is very much reduced, but the oxidation of the acetoacetic and β-hydroxybutyric acids still occurs.

According to the theory of antiketogenesis supported by Shaffer [1921] the oxidation of the ketone bodies by the tissues can only proceed after molecular union with some carbohydrate derivative has taken place.

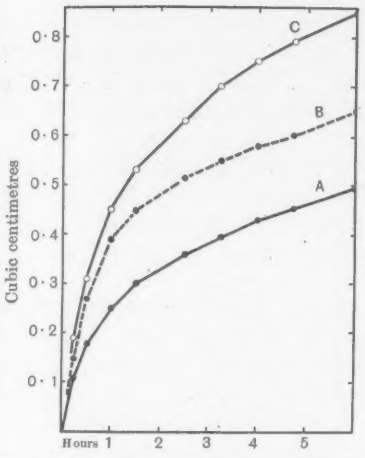

Fig. 1. Uptake of oxygen by 0·5 g. quantities of liver preparation from normal rat. *A*, liver alone. *B*, liver plus β-hydroxybutyric acid. *C*, liver plus acetoacetic acid.

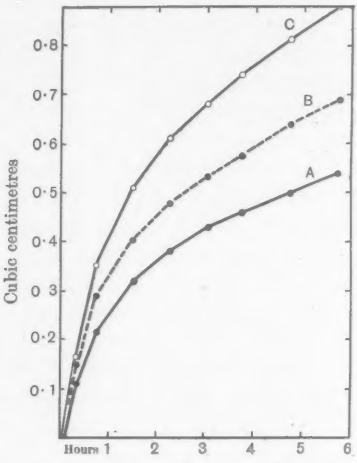

Fig. 2. Same as Fig. 1. Rat, fasted for 36 hours.

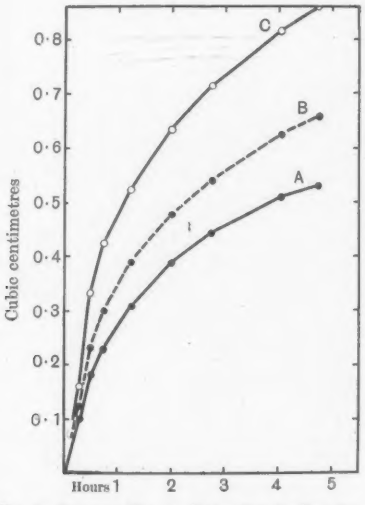

Fig. 3. Same as Fig. 1. Rat, after feeding for 3 days on butter fat alone.

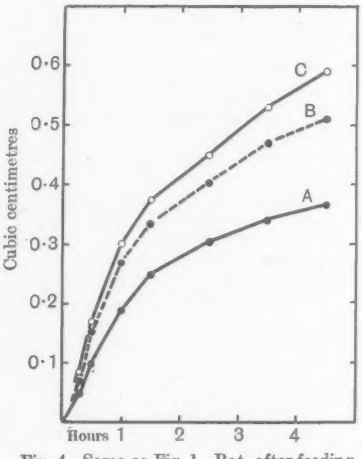

Fig. 4. Same as Fig. 1. Rat, after feeding for 10 days on butter fat alone.

An attempt was made to test this theory by experiments carried out on the lines described. Since these experiments have yielded uniformly negative results, they can be referred to very briefly.

Glucose added to the fresh liver preparation in a concentration of 1/25th molecular does not lead to an increased oxygen uptake; nor is the oxidation of the ketone bodies increased by the presence of glucose under these conditions.

Shaffer considers that the "catalysis" of the oxidation of acetoacetic acid by hydrogen peroxide in alkaline solution *in vitro* is due to its union with some oxidation product of the glucose. Since the main product of the action of hydrogen peroxide on glucose is glucosone, the above experiments were repeated with this substance. Glucosone was prepared by the method of Fischer [1889], and added in about the same concentration as was the glucose.

Glucosone caused no increase in the oxygen uptake, and it did not increase the oxidation of acetoacetic acid or β-hydroxybutyric acid.

A similar experiment was carried out with a solution of glucose which had been partially oxidised by the addition of one molecular equivalent of hydrogen peroxide in the presence of 1: 10,000 Fe and allowing to stand for twelve hours. Such a solution of mixed oxidation products of glucose caused a very marked increase in oxygen uptake. The uptake during four hours by the controls was 500 c.mm. of oxygen and in the presence of the above mixture 1079 c.mm. This increase was not due to the iron present, which alone in the same concentration caused a doubtful increase of about 10 %. The oxidation of the ketone bodies was not increased in the presence of these mixed oxidation products of glucose.

It has been suggested that before glucose can take part in metabolic changes it must first be "activated," and that such activation may be equivalent to the "dissociation" of glucose by alkalies [Woodyatt, 1915]. An 8 % solution of glucose was therefore treated with 10 % sodium hydroxide at room temperature for fifteen hours, neutralised to p_H 7·4 with hydrochloric acid and the same experiment repeated. Such a solution of partially broken down glucose caused a very slight increase in oxygen uptake (10 %) but did not augment the oxidation of the ketone bodies.

Woodyatt has further suggested that in the body one of the functions of the internal secretion of the pancreas is to activate glucose so that it can enter into combination with the ketone substances. The same technique has been used to investigate the question. Sections of the fresh pancreas of the rat were cut under the same conditions as those of the liver and added to the liver preparation. The oxidation of acetoacetic acid and β-hydroxybutyric acid was not increased in the presence of pancreas, either alone or with the addition of glucose.

The experiments were repeated using insulin in place of fresh pancreas and with the same results.

Thus under no experimental conditions to which the living rat or its tissues

after removal from the animal have been subjected could the oxidation of the ketone bodies by the liver be definitely influenced. If the liver is indeed the main locus of the breakdown of the ketone bodies and if this oxidation which has been studied is really the main oxidative process which they undergo, then in so far as the experiments throw any light on the phenomenon of ketosis they tend to support the view that ketosis is due to an increased production of ketone bodies rather than to a diminished oxidation; or that possibly their normal fate is synthetic rather than oxidative and that this change is interfered with.

It is of interest that acetoacetic acid appears to be more readily oxidised by the liver of the rat than does β-hydroxybutyric acid in the same molecular concentration. If, as is generally held, the normal conversion of these substances is from acetoacetic acid to β-hydroxybutyric acid it was to be expected that the latter would be the more readily oxidised even when the inactive acid was used. This difference in the oxidisability of the two acids may possibly have some bearing on the relative concentrations in which they appear in the urine during alkalosis in the rat.

SUMMARY.

A technique is described by which accurate sampling of fresh tissue is obtained for comparative measurements of oxygen uptake.

The fresh liver of the rat shows an increased uptake of oxygen in the presence of acetoacetic acid and inactive β-hydroxybutyric acid. When present in the same molecular concentrations the former causes the more marked increase.

The oxidation of the ketone bodies by the liver does not appear to be affected by fasting the animal for thirty-six hours, nor at any stage in the adaptation to a diet of fat.

The oxidation of the ketone bodies is not affected by the presence of glucose or its breakdown products, nor by the presence of pancreatic extracts or insulin.

It is suggested that ketosis is not due to a diminished direct oxidation of ketone bodies.

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CLVII. A NOTE ON THE SOLUBILITIES OF CALCIUM SOAPS.

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(*Received August 12th, 1924.*)

In the course of some special work it was necessary to know the solubilities and melting points (if any) of calcium oleate, palmitate, and stearate. The information in standard works of reference was meagre and to some extent contradictory, and a summary of it is given below (Table I). No statement as to the existence or otherwise of a melting point for any of the three soaps was found.

Table I.

Authority	Calcium oleate	Calcium palmitate	Calcium stearate
Beilstein [1920]	Soluble in alcohol and ether	100 parts absolute alcohol dissolve at 20°, 0·0103 part	No data
Lewkowitsch and Warburton [1921]	Insoluble in alcohol and ether. 200 cc. of a 10 % sugar solution, after digestion with oleate for 1 hour, dissolve at 19·5, 35, 50 and 65°, 0·0661, 0·1767, 0·450 and 0·726 g. respectively. Solution in pyridine passes through an india-rubber membrane	100 parts absolute alcohol dissolve at 20°, 0·0103 part	Practically insoluble in alcohol
Ullmann [1920]	Insoluble in alcohol and ether	No data	No data
Seidell [1920]	1000 cc. water dissolve about 0·1 g. at $t°$ not stated (Fahrion). 100 g. glycerol (of $d=1·114$) dissolve 1·18 g. at $t°$ not stated (Asselin)	No data	No data
The Condensed Chemical Dictionary [1919]	No data	No data	Soluble in alcohol and ether: insoluble in water
Martindale and Westcott [1920]	Slightly soluble in alcohol and ether	No data	No data

The three salts were prepared by "double decomposition," and the details given in Table II were ascertained by direct experiment.

Method of Preparation.

A known weight of the organic acid (oleic, palmitic or stearic) was dissolved in absolute alcohol and neutralised with caustic soda (10 % aqueous solution), using phenolphthalein as indicator.

A calculated excess of calcium chloride was dissolved in a large volume of water, and the sodium salt, which had been prepared as above, was added. The precipitate of the calcium soap was washed repeatedly on a Buchner filter with water, and lastly with absolute alcohol. The soap was finally dried in a vacuum desiccator over calcium chloride.

Table II.

	Calcium oleate			Calcium palmitate			Calcium stearate		
Solvent at	0°	Room temp. 15–20°	B.p. of solvent	0°	Room temp. 15–20°	B.p. of solvent	0°	Room temp. 15–20°	B.p. of solvent
Alcohol (ethyl, absolute)	Insol.	Insol.	Insol.	Insol.	Insol.	Insol.	Insol.	Insol.	Sl. sol.
Ether (di-ethyl)	„	„	„	„	„	„	„	„	Insol.
Chloroform	V. sl. sol.	Fairly sol.	Sol.	„	V. sl. sol.	Sl. sol.	„	„	V. sl. sol.
Acetone	Insol.	Insol.	Insol.	„	Insol.	Insol.	„	„	Insol.
Benzene	Sl. sol.	Fairly sol.	Sol.	„	V. sl. sol.	Sl. sol.	„	V. sl. sol.	Sl. sol.
Xylene	„	Fairly sol.	„	„	Insol.	V. sl. sol.	V. sl. sol.	Sl. sol.	Fairly sol.
Light Petroleum (b.p. 50–60°)	Insol.	Insol.	Insol	„	„	Insol.	Insol.	Insol.	Insol.
Water	„	„	„	„	„	„	„	„	„
Effect of heating:	No definite m.p. Conglutinates 115–120° Starts to decompose 140–160°			No definite m.p. Conglutinates 110° Starts to decompose 150–155°			No definite m.p. Conglutinates 115–120° Starts to decompose 155–160°		

Abbreviations: Insol. = insoluble. Sl. = slightly. V. sl. = very slightly. Sol. = soluble. M.p. = melting point. B.p. = boiling point.

Notes: All three salts may be "dissolved" by acid alcohol (we used 1 % HCl in absolute alcohol)—slightly at 0°, with fair ease at room temperature (15–20°), and readily when the acid alcohol is boiling.

Calcium stearate was obtained as anisotropic "osazone-like" sheaves by crystallising from hot alcohol. It was necessary to allow the solution to cool very slowly.

It is felt that the above data (Table II), though only qualitative, may possibly be of some value to other workers.

I am much indebted to my assistant, Mr R. J. Bromfield, for much of the labour in the above observations.

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CLVIII. A METHOD FOR THE ESTIMATION OF SMALL QUANTITIES OF COPPER IN TISSUES.

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(*Received August 13th, 1924.*)

THE common microchemical method for the determination of copper in tissues is that devised by Rose and Bodansky [1920], who have investigated the quantitative distribution of this element in mammalia, in fish, and in crustacea. Their method which is colorimetric depends on the production of a blue colour by the interaction of ammonium hydroxide and $CuSO_4$ in aqueous solution.

Theoretical.

The present method is based on the development of a light green colour, "Schcele's Green," when a solution of copper sulphate is added to a solution of Na_3AsO_3. The equation representing the reaction is:

$$Na_3AsO_3 + CuSO_4 + H_2O = CuHAsO_3 + Na_2SO_4 + NaOH \quad ...(1).$$

"Scheele's Green" is copper hydrogen arsenite.

We found that anhydrous arsenic chloride dissolved in dilute caustic soda is an extremely colour-sensitive reagent. Presumably these two substances react in solution in the following manner:

$$AsCl_3 + 6NaOH = Na_3AsO_3 + 3NaCl + 3H_2O \quad .....(2).$$

The Na_3AsO_3 then reacts as shown in equation (1).

Practical.

To prepare the reagent 10 cc. of 10 % NaOH are taken in a boiling tube and 0·1 cc. of anhydrous $AsCl_3$ added. A white precipitate which disappears on shaking is formed. A second 0·1 cc. $AsCl_3$ is now added, and the boiling tube is again shaken. The contents of the tube are now diluted by the addition of 10 cc. of distilled water. The reagent is now ready for use.

An experiment is described illustrating the colour proportionality to copper concentration:

0·8472 g. of $CuSO_4$ was dissolved in distilled water and made up to 1 litre. 3, 4, 5, 6 cc. respectively of this solution were transferred to four graduated cylinders of 15 cc. capacity, and distilled water was added to each cylinder until the volume was 10 cc. Each cylinder was stirred with a short glass rod, which was left in the solution.

Five drops of the $AsCl_3$ reagent were now added to each cylinder, and the green colour developed instantaneously. The contents of the cylinder containing 3 cc. of standard $CuSO_4$ solution were transferred to the colorimeter cup, and the other solutions were read against this as standard.

The following table (Table I) gives the results obtained and from these a curve can be drawn showing the relationship between colour intensity and concentration of $CuSO_4$. This curve is approximately a rectangular hyperbola.

Table I.

Concentration in 10 cc.	0·0025416 g.	0·0033888 g.	0·0042360 g.	0·0050832 g.
Colorimeter reading	2·32	1·56	1·26	1·08

The determination of the copper content of ox brain was made by this method. The copper present was converted to copper sulphate. To this end, a portion of brain (100 g.) was subjected to a process of moist combustion, and the inorganic residue was extracted with hot water containing a trace of dilute HNO_3. The filtrate from the extract was evaporated to dryness, taken up with hot dilute HCl, filtered, and the filtrate treated with H_2S. After filtration, the copper sulphide residue was dissolved in hot dilute HNO_3 and the copper converted into oxide by heating on the microburner after evaporation to dryness. Two drops of 0·1 % H_2SO_4 were added and the contents of the basin transferred to a graduated cylinder, and made up to a volume of 6 cc. with distilled water. The standard was made by taking 1 cc. of $CuSO_4$ solution (1 cc. = 0·0008472 g. $CuSO_4$) and diluting to 6 cc. The following were the colour readings:

Reading of Unknown = 1·26.

,, Standard = 0·55.

$\therefore$ Weight of $CuSO_4$ in 100 g. of brain $= \frac{0{\cdot}55}{1{\cdot}26} \times 0{\cdot}0008472$ g. $= 0{\cdot}0003697$ g.

$\therefore$ Percentage of Copper = 0·0000935.

Discussion.

The arsenic chloride reagent here used is sensitive to 1 in 135,000 of $CuSO_4$ in water. It can be stored in a well-stoppered bottle without appreciable deterioration, and gives the minimum of trouble in manipulation. Its limits of efficiency are, however, circumscribed, the upper limit of $CuSO_4$ concentration for which it will give reliable results being ($6 \times 0{\cdot}0008472$ g.) in 10 cc. H_2O. If the concentration is very much increased beyond this point, there is a tendency towards sedimentation of a green substance. This can be obviated by the graduated addition of 10 % NaOH solution, but excess of caustic soda is apt to cause decolorisation. Between the limits S and $6S$ (where $S = 0{\cdot}0008472$ g. $CuSO_4$ in 10 cc. H_2O), the colour is permanent and there is no centrifugable compound present. There is, of course, a colour permanence below the limit S, but the thinness of colour developed under this concentration is such as renders

it not very suitable for colorimetric comparison. Above a concentration 6*S* a nephelometric technique is necessary.

The coloured compound produced is thermolabile. On warming a test tube containing the green coloured substance in concentration ranging between *S* and 6*S* there is an immediate separation of a solid green substance. It is, therefore, important that tissue extracts be treated in the cold with the reagent.

The presence of iron and magnesium is inimical to the straightforward determination of copper, as the salts of these metals interfere with colour development. These two elements are easily separable from copper in the early process of extraction as above described, and the method therefore requires no elaboration here.

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CLIX. GASTRIC UREASE.

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(*Received August 15th, 1924.*)

As one of us [Luck, 1924] has recently shown that the enzyme urease occurs in the gastric mucosa of the Carnivora and in specified portions of the stomachs of the Ruminantia, it was considered desirable to compare "animal" urease from the gastric mucosa of the dog, with "plant" urease from the soy bean. Two possibilities suggested themselves:

1. Unlike soy bean urease, gastric urease might be able to catalyse the dissociation and hydrolysis of ureides, and thus contribute to the processes of gastric digestion.

2. Gastric urease might be an endocellular enzyme, capable only of catalysing the dissociation of urea, and taking part in neutrality regulations within the body, by converting urea from the blood into ammonium carbonate, or by synthesising urea from absorbed ammonia.

The examination of the first of these hypotheses has been the object of this investigation. The action of gastric urease on many urea derivatives, and on several amides has been investigated. Most of the substrates employed have been examined by previous workers with soy bean urease, but were re-investigated in a parallel duplicate series. As uramino acids have at times been isolated from urine, and have been frequently considered as true metabolites, the uramino acids of glycine and leucine were prepared and subjected to the action of soy bean urease and gastric urease.

The results of this portion of the work indicate that gastric urease is identical with soy bean urease.

It has been shown [Van Slyke and Zacharias, 1914; Lövgren, 1921, 1923] that the optimum hydrogen ion concentration for the action of soy bean urease varies with the concentration of the substrate. Van Slyke and Zacharias found an optimum p_H of 7·7 for 0·02 molar urea and p_H 7·0 for 0·15 molar urea. Lövgren reported an optimum p_H of 7·70 for 0·01 molar urea, 7·45 for 0·1 molar and 7·20 for 1·6 molar. It was also shown by the latter author that the optimum hydrogen ion concentration for soy bean urease was not influenced by the phosphate concentration, the enzyme concentration, or the reaction time. As this dependence of the optimum p_H on

substrate concentration has not to the authors' knowledge been demonstrated with other enzymes, it appeared desirable to determine the optimum p_H for the action of gastric urease and observe its dependence, if any, on the substrate concentration. These results also demonstrated that like soy bean urease, the optimum p_H for the action of gastric urease increases with dilution of the substrate. It is therefore considered that gastric urease is identical with soy bean urease and that the first of the above hypotheses is untenable.

Experimental.

(1) *Preparation of the enzyme from soy bean meal and from the gastric mucosa of the dog.*

The method of Van Slyke and Cullen [1914], applied to aqueous extracts of the gastric mucosa of the dog, yielded preparations of low solubility and of only moderate activity.

Various modifications were tried and eventually a successful preparation was obtained by grinding the freshly stripped mucosa with much sand, desiccating the mixture *in vacuo* and extracting the dry powder by thorough grinding with water. The latter was added in the proportion of 10 parts of water to 1 part of freshly stripped mucosa. Assuming the water content of the fresh tissue to be 80 %, the treatment amounted to the extraction of 2 parts of dry tissue with 108 parts of water. The aqueous solution obtained on subsequent centrifuging was slightly opalescent and of high activity, being slightly more active than the solution obtained by grinding 2 parts of soy bean meal with 108 parts of water. The dried product retains its activity almost undiminished for many weeks. In the experiments which follow, fresh aqueous extracts were prepared from the dried product daily. The soy bean preparation was similarly obtained by grinding 2 parts of the meal with 108 parts of water, and centrifuging off the aqueous extract.

(2) *Technique.*

The amount of ammonia produced in 15 minutes at 38° by 1 cc. of the enzyme solution, acting on a fixed amount of urea in the presence of a fixed amount of phosphate solution was taken as an index of the activity of the enzyme.

Ammonia was estimated by Van Slyke and Cullen's [1914] aeration method.

Phosphate buffer solutions were prepared by mixing in requisite amounts molar solutions of dihydrogen potassium phosphate and monohydrogen potassium phosphate [Lövgren, 1923]. This series of solutions was then standardised against the hydrogen electrode.

(3) *The optimum* p_H *for the action of gastric urease.*

(i) 1 cc. of enzyme solution at 38° was added to 9 cc. of a solution at 38° consisting of 5 cc. of urea (0·04 molar), 1 cc. water, 3 cc. molar phosphate solution of given p_H. The tubes were corked. After exactly 15 minutes, the

reaction was stopped by the addition of caprylic alcohol and potassium carbonate in the usual manner. This series of experiments was performed in duplicate on the same day with the same enzyme solution. The duplicates were always observed to be in close agreement. The average values have been plotted in Fig. 1, Curve (*a*). The optimum p_H for gastric urease in 0·02 molar urea was observed to be about 8·1.

(ii) On a second day the observations were repeated with 5 cc. of 2*M* urea. The results are presented in Fig. 1, Curve (*b*). The optimum p_H for gastric urease in molar urea was observed to be about 7·5.

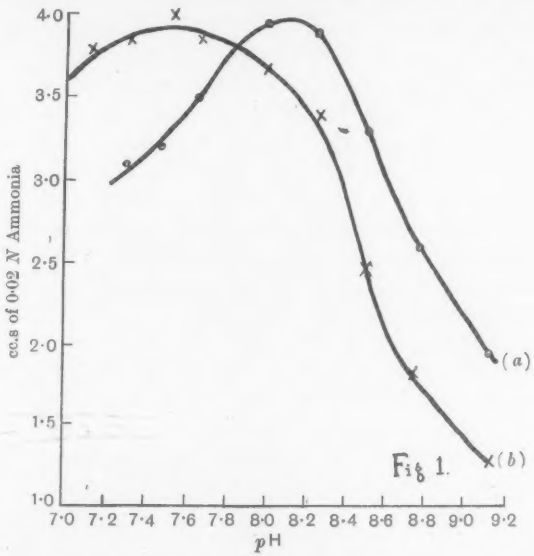

Fig. 1

These observations regarding the dependence of the optimum p_H for gastric urease on the substrate concentration are in entire agreement with the observations of Van Slyke and Zacharias [1914] and Lövgren [1923] on soy bean urease. While the optima observed by us are in general higher than those reported for similar concentrations of urea with soy bean urease, it is suggested that the vast differences in the composition of the extraneous matter associated with the enzyme in each case may cause this apparent difference. The fundamental fact is that the optimum p_H of both gastric urease and soy bean urease varies with the concentration of the substrate.

(4) *The specificity of gastric urease.*

The "specificity" of soy bean urease is one of its most distinctive features and in order to determine the identity or otherwise of gastric urease and soy

bean urease the action of the two preparations on a large number of substrates allied in composition to urea was studied under comparable conditions.

The enzyme solutions were prepared as described in section (1).

Duplicate experiments with suitable controls were carried out with each enzyme preparation, using in each case:

5 cc. of 0·04 molar substrate.
3 cc. of molar phosphate (p_H 7·99).
1 cc. of water.
1 cc. of enzyme solution.

The reaction in each case was allowed to proceed for two hours at 39–40°, after which ammonia was estimated in the usual manner. The following substrates were investigated:

Urea, monomethyl urea, *unsym.* dimethyl urea, *sym.* dimethyl urea, monoethyl urea, *unsym.* diethyl urea, *sym.* diethyl urea, normal butyl urea, phenyl urea, monoacetyl urea, thio-urea, biuret, urethane, creatine, acetamide, propionamide, benzamide, succinamide, succinimide, asparagine, oxamic acid, oxamide, adenine sulphate, guanine chloride, uramino-acetic acid and *α*-uramino-isobutylacetic acid. The last two were prepared by the method of Lippich [1908] from glycine and leucine respectively.

All the above substrates except urea and normal butyl urea gave negative results. The observations of Armstrong and Horton [1912] (negative results), Werner [1923] (negative), Takeuchi [1909] (negative), Wester [1921] (negative), and Fearon [see Werner, 1923, p. 110] (positive results with normal butyl urea) are confirmed. The positive results of Takeuchi with biuret, and of Pin Yin Yi [1920] with *unsym.* dimethyl urea, and *unsym.* diethyl urea (using Robinia urease) could not be reproduced. The results obtained with urea and butyl urea follow:

Substrate	Enzyme	Time	Nett cc. $N/50$ NH_3
Urea	Gastric urease	15 min.	4·45
			4·55
„	Soy bean urease	15 „	3·90
			3·95
Butyl urea	Gastric urease	2 hours	0·90
			0·95
„	Soy bean urease	2 „	0·82
			0·85

These results suggest that the two enzymes are identical.

Urea is probably the sole metabolite which is capable of functioning as substrate for the gastric enzyme. The ammonia of the gastric juice, 2 mg. per 100 cc. [Huber, 1917], is 50 to 100 times more concentrated than the ammonia of the arterial blood. We would suggest that the gastric juice ammonia arises at least in part from the localised action of gastric urease on urea from the blood.

The second hypothesis is now under investigation.

SUMMARY.

1. Gastric urease from the mucosa of the stomach of the dog has been compared with soy bean urease.

2. The common dependence of the optimum p_H on substrate concentration, the common specificity of the two enzymes, and their similar action on butyl urea, suggest that gastric urease and soy bean urease are identical.

3. A partial explanation of the origin of the gastric juice ammonia is proposed.

A number of the substituted ureas were very generously supplied by Professor E. A. Werner of Trinity College, Dublin. The supervision of Professor F. G. Hopkins is also gratefully acknowledged.

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CLX. THE ESTIMATION OF GLUCOSE IN PHYSIOLOGICAL FLUIDS BY THE REDUCTION OF COPPER SALTS.

By WILLIAM WHITE TAYLOR.

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(*Received August 22nd, 1924.*)

The importance of the estimation of glucose in physiological fluids does not need to be emphasised; that there are difficulties in connection with the estimation is proved by the many variations and modifications of the copper reduction method which have been introduced from time to time[1].

It is a commonplace among teachers of physiological chemistry that a normal urine, to which glucose has been added, cannot be titrated satisfactorily with Fehling's solution, the end-point being much more indefinite than with diabetic urine; and attempts, unsuccessful so far as I am aware, have been made to ascertain to what substances, either by their presence or absence, the difference is due. It occurred to me that there might not be the same difficulty with reagents of the type of Benedict or Folin-McEllroy, which give a white precipitate of cuprous thiocyanate, instead of red cuprous oxide.

On trial this was found to be the case, the end-point being easily observed, though the liquid is yellowish, and not colourless as with pure glucose solutions.

But when a known amount of glucose solution was added to normal urine (*i.e.* no trace of reduction of Folin-McEllroy qualitative reagent, no albumin, bile, or ketone bodies), the amount of glucose indicated by the titration against Folin-McEllroy quantitative reagent was always greater than the amount actually added. This discrepancy is not peculiar to the Folin-McEllroy reagent, for similar results are obtained with Benedict's reagent, and also with Fehling's standard reagent.

The following results are typical:

	5 cc. of 2 % glucose added to	
Reagent	20 cc. water	20 cc. urine
Folin-McEllroy (5 cc.)	6·80 cc. 6·70 „ 6·75 „ } (=0·370 %)	4·95 cc. 5·20 „ 5·15 „ } (=0·485 %)
Benedict (10 cc.)	5·40 „ (=0·370 %)	4·15 „ (=0·482 %)
Fehling (5 cc.)	5·8 „ (=0·43 %)	4·6 „ (=0·54 %)

[1] The principle and theoretical basis of the method and of the modifications recently introduced, are so well known that it is not necessary to give a review of them, nor of the literature relating to them.

The difference in the two values is over 30 %.

A further preliminary experiment was done to ascertain whether a similar effect was obtained with diabetic urine. The result was as follows:

	5 cc. of Folin-McEllroy reagent.		
(*a*)	Diabetic urine	3·65 cc.	(= 0·685 %)
(*b*)	,, ,, (10 cc. + 1 cc. of 2 % glucose)	2·90 cc.	(= 0·862 %)

The calculated value for (*b*) is 0·804 %.

That the divergence is not constant, but depends on the amount of urine added to a glucose solution is shown by the figures given below:

5 cc. of a 2·5 % solution of glucose were made up with the specified amounts of urine and water to a total volume of 25 cc. and titrated against 5 cc. of Folin-McEllroy reagent.

Urine	Water	Titration	Glucose (%)
—	To 25 cc.	4·92	0·508
1 cc.	,,	4·35	0·575
2 ,,	,,	3·97	0·630
To 25 ,,	—	3·65	0·685

The final increase is 35 % which agrees with the 31 % increase previously found, considering that different samples of urine were used in the two experiments.

Of the various known constituents of normal urine, uric acid and creatinine appeared most likely to be the cause of these differences; for, though they, either alone or together, may not be capable of oxidation by the copper reagent used, it is quite possible that when glucose is being oxidised in their presence, they also undergo oxidation. It is also possible that these or other constituents of the urine enter into combination with part of the copper, forming a complex which is not so readily reduced as the original copper complex.

The Effect of Uric Acid.

In the experiments the results of which are given below, the solutions used were 0·5 % uric acid, dissolved by the aid of lithium carbonate, and 0·5 % glucose. The stated amount of uric acid was added to 5 cc. of Folin-McEllroy reagent and the titration carried out in the usual manner. The table gives a summary of the results obtained.

5 cc. of Folin-McEllroy reagent		
Uric acid (0·5 %)	Glucose (0·5 %)	Decrease in cc. for 0·1 cc. uric acid
—	4·98 cc.	—
0·2 cc.	4·90 ,,	0·040
0·3 ,,	4·85 ,,	0·043
0·4 ,,	4·78 ,,	0·050
0·6 ,,	4·66 ,,	0·054
0·8 ,,	4·59 ,,	0·049
1·0 ,,	4·48 ,,	0·050
1·5 ,,	4·29 ,,	0·046
2·0 ,,	4·15 ,,	0·042

The figures in the third column are obtained by subtraction of the corresponding titration values from that of glucose alone, and division of this difference by the number of 0·1 cc. added. They show that the effect produced is practically proportional to the amount of uric acid added.

The Effect of Creatinine.

The solutions used in the following determinations were 0·320 % creatinine, 0·51 % glucose, the procedure being the same as with uric acid.

The next table gives a summary of the results.

5 cc. of Folin-McEllroy reagent		
Creatinine (0·320 %)	Glucose (0·51 %)	Decrease in cc. for 0·1 cc. creatinine
—	4·86 cc.	—
0·05 cc.	4·84 „	0·04
0·10 „	4·66 „	0·20
0·15 „	4·55 „	0·21
0·20 „	4·58 „	0·14
0·25 „	4·55 „	0·12
0·50 „	4·60 „	0·05
1·0 „	4·45 „	0·04

One difference between the uric acid and the creatinine results is at once apparent. With the latter the figures in column 3 are not even approximately constant; in fact after 0·15 cc. there is practically no change in the titration value until 1 cc. has been added.

Another difference, equally apparent when carrying out the estimations is, that creatinine, even in small amounts, retards the reduction to such an extent that it is not easy to complete the titration within the prescribed eight minutes. Only when almost the whole of the glucose required in any given case, as found by a preliminary titration, is added at the beginning, and the solution then kept boiling for at least six minutes (instead of the usual four minutes) is it possible to finish in the time, and obtain concordant results.

The colour of the reagent is distinctly changed to a greenish blue on addition of the creatinine, and the final change is from a greenish tint to colourless.

The Effect of Urea and Ammonium Salts.

When a brief account of the preliminary observations on this subject was presented at the International Physiological Congress, Professor Emil Werner suggested that it would be interesting to know whether ammonium salts were also oxidised along with the glucose by the copper reagent. For he had found in his investigation [Werner, 1922] of the decomposition of urea by alkaline hypobromite, that when glucose is added to the urea, as recommended by Méhu [1879] the volume of gas evolved is increased, although the formation of cyanate is not prevented, and is in fact increased; the increase in volume of gas is due to the production of carbon monoxide from the glucose, although

none is evolved when glucose alone is added to alkaline hypobromite. Further, when ammonium chloride, to which glucose is added, is acted on by alkaline hypobromite, the volume of gas evolved progressively diminishes with increasing amounts of glucose, though it contains carbon monoxide, while the amount of cyanate progressively increases. This point will be referred to later.

As a matter of fact, it has been found that the presence of ammonium salt has no marked effect on the estimation of glucose by means of the Folin-McEllroy reagent and the same applies in the case of urea.

The Effect of Creatinine + Uric Acid.

Since urea and ammonium salts were found to have no appreciable effect, it remained to ascertain how far the presence of both creatinine and uric acid affected the titration. The results are given below.

Creatinine + Uric Acid.

5 cc. of Folin-McEllroy reagent

Creatinine (0·320 %)	Uric acid (0·5 %)	Glucose (0·5 %)	Difference	
			Obs.	Calc.
—	—	4·94	—	—
0·2 cc.	—	4·66	0·28	—
—	0·8 cc.	4·64	0·30	—
0·2 „	0·8 „	4·60	0·34	0·58
0·2 „	0·8 „	4·58	0·36	
0·1 „	—	4·72	0·22	—
0·1 „	0·8 „	4·60	0·34	0·52

It is evident that the effect is not additive, although it is greater than that of either alone. The retardation of reduction was very marked; indeed it was almost impossible to complete the titration in less than 10–12 minutes, and this may affect the end-point of the reaction. Of the duplicate estimations given above, the first took eleven minutes, the second between eight and nine minutes, as almost the whole amount was added at once.

Discussion of Results.

The experiments described above have established that when glucose is oxidised in urine, or in the presence of uric acid, or creatinine, by means of alkaline copper reagents, the reducing action is greater than when the same amount of glucose in water is treated with the same reagent.

Regarding the mechanism of the reaction, three possible explanations suggest themselves:

(1) Coupled reaction: *i.e.* the copper reagent which does not appreciably oxidise the uric acid or creatinine when alone, does so when glucose is oxidised in their presence. Many such coupled reactions are known.

(2) The glucose is oxidised in a different manner or to a different stage in the presence of these substances, which themselves do not undergo oxidation.

(3) The substances enter into combination with the copper reagent, forming complexes which resist reduction.

Which of these explanations is the most probable, the evidence available at present does not enable one to decide. In favour of (3) one may point to the colour change when creatinine is added, and to the marked retardation of the reduction. There is also the fact that after the addition of a small amount of it, subsequent increase in the amount produces little or no further change. It should be borne in mind that all the copper reagents in use contain fairly stable complexes, and that whether tartrate, citrate, phosphates, or carbonates are used, reduction takes place quite readily.

Many experiments were done on the retarding effect of these substances on Folin-McEllroy qualitative reagent, the amount of glucose being added which alone just produced a yellow precipitate in three minutes in a boiling water bath. Both creatinine and uric acid delayed the first change in colour, but the order of apparent maximum precipitation was not the same at different intervals of time.

According to Werner [1922] no carbon monoxide is evolved when glucose is acted on by alkaline hypobromite (at least not in a reasonable period of time), whereas when ammonium chloride is present, considerable amounts of carbon monoxide are evolved along with the nitrogen. According to my experiments, ammonium sulphate has a negligible effect; and besides, unlike Werner's case, here it is the glucose which is the active reducer, as ammonium salt alone is not appreciably oxidised by the copper reagent.

It was thought that some direct evidence of a coupled reaction might be obtained in the case of ammonium salt. As Werner had found that cyanate is always produced when ammonia is acted upon by alkaline hypobromite in the presence of glucose, he suggested that cyanate would probably be found in my experiments with ammonium salts. But though Werner's copper-pyridine-chloroform test for cyanate is very delicate, I failed to detect any cyanate in reductions of Folin-McEllroy qualitative reagent (which contains no thiocyanate) by glucose in presence of ammonium sulphate. Further experiments on a larger scale are necessary.

A further point requires consideration: in the type of glucose estimation investigated a fixed amount of copper reagent is completely reduced by gradual addition of the reducing solution. And it is clearly established that it is inadmissible to calculate glucose from the titration value, when uric acid and creatinine are also present. It is a matter of importance to know whether the same applies to methods in which an excess of copper reagent is treated with variable amounts of glucose.

Some preliminary experiments have been made on Folin's micro-method, and these indicate that creatinine and uric acid have a distinct effect, as was indeed to be expected. For, in the first place, the prescribed time of immersion in boiling water is *exactly* six minutes for both the standard and the unknown. It is soon found that if the time is increased, the reduction is also increased, *e.g.* identical solutions (Folin standards) heated for six, nine and twelve minutes gave colorimeter readings of 20, 19·2, 18·2. When a small amount of

creatinine is added, the retardation predominates and the glucose estimation is correspondingly low, when the time of heating is six minutes; while, if heated for ten minutes the increased reduction comes in, and the estimated glucose is higher, *e.g.*

		6 min.	10 min.
Folin standard		20·0 mm.	17·7 mm. (6 min. $S=20$)
„	+0·1 cc. Creatinine	22·6 „	16·3 „

Further work on this and other methods in which excess of copper reagent is used are in progress.

Summary.

1. A glucose solution diluted with normal urine has a greater reducing power on Folin-McEllroy copper reagent (also on Benedict and Fehling) than when diluted with water. The difference may amount to over 30 %.

2. Creatinine and uric acid (and to a practically negligible degree ammonium salt) produce the same effect. Urea has no action.

3. Creatinine causes a marked retardation of the reduction.

4. There are indications that the methods of glucose estimation, in which excess of copper reagent is used are similarly affected by uric acid and creatinine.

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CLXI. AN UNUSUAL TYPE OF FATTY COMPOUND IN A PRODUCT OF ANIMAL ORIGIN.

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Some time ago one of us [Funk, 1923] reported the presence of a fatty substance of M.P. 52° in all samples of caseinogen, except those which have been subjected to prolonged extraction with boiling alcohol. Extraction with ether apparently does not remove this substance to any extent. It was thought at first that this substance might be of biological importance, but specially conducted feeding experiments on rats proved that this was not so. However, on close investigation, the substance proved to be a rather peculiar combination of fatty acids. Neutral in nature, it could be regarded as a glyceride, but on saponification it yielded only a fatty acid, in slightly higher yield than the original substance taken. The fatty acid is probably one of the hydroxystearic acids, two molecules of which form an anhydride. On account of difficulty encountered in purification, and a rather low melting point for such a high-carbon acid, we consider it not impossible that our purest product is contaminated with the next homologue, hydroxyarachidic acid. From the analytical figures it is even possible that we are dealing with a mixed anhydride of these acids.

Erben [1900] has reported the isolation from the urine of a case of chyluria of a substance of M.P. 51·5°, which he regarded as a mixture of hydroxystearic acids. Lanocerin, according to Röhmann [1906], is an anhydride of lanoceric acid, a hydroxy acid.

Experimental.

Caseinogen is repeatedly extracted under a reflux condenser with boiling alcohol, and on cooling our fatty substance separates out. The combined yield reaches 1·5 %. On recrystallisation from alcohol, a small insoluble residue remains behind, and the substance is obtained in the crystalline state, with an indefinite melting point of about 49°. After repeated crystallisation from ether and ethyl acetate the substance melted at 55–55·5° and solidified at 44·5°. The product is entirely neutral and is rather insoluble in cold alcohol. All analyses were made according to Pregl's[1] micro-methods.

[1] We take this opportunity to thank Prof. Pregl for his hospitality shown to one of us (C. F.) in his laboratory and to Mr W. H. F. Kuhlmann of Hamburg for a prompt delivery of one of his excellent micro-balances.

Two analyses gave 12·48 % H, 73·90 % C, and 12·05 % H, 74·45 % C. Calculated for anhydride of hydroxystearic acid $C_{36}H_{70}O_5$ (582·56), 74·15 % C and 12·11 % H.

A determination of the molecular weight was made according to the micro-method of Rast [1922]. Two determinations gave 580·5 and 630.

Saponification.

One gram of substance was saponified with 20 % alcoholic KOH. Yield of acid obtained 1·01 g. The product was recrystallised from 70 % alcohol and benzene to avoid esterification and gave colourless glistening plates, which melted at 57·5–58° and solidified again at 55·5°. The acid is much more soluble in alcohol than the anhydride. Determination of acidity gave higher figures than calculated, probably due to the interference of the hydroxy-group. For the preparation of the silver salt, the acid was dissolved in hot absolute alcohol and a boiling alcoholic silver nitrate solution was added. Some of the product separates out immediately, the rest on cooling. The crystals were filtered by suction, washed out with cold alcohol and then repeatedly with water.

Three analyses of the silver salt gave 26·36, 27·10 and 26·61 % Ag. Calculated for $C_{18}H_{35}O_3Ag$, 26·50 % Ag.

The free acid gave the following figures on combustion: C 71·96, H 12·16; C 71·76, H 12·30. Calculated for $C_{18}H_{36}O_3$ (300·29), 71·93 C and 12·08 H.

The molecular weight determination gave 302.

We could not identify the acid with any of the described hydroxystearic acids. The anhydride found in the caseinogen is unlikely to be formed secondarily, as prolonged boiling of the acid with alcohol did not yield any anhydride.

In studying the fats it seems to be worth while to pay attention to these anhydrides, which resemble very closely the ordinary glycerides of fatty acids.

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CLXII. ALLELOCATALYTIC EFFECT IN CULTURES OF *COLPIDIUM* IN HAY-INFUSION AND IN SYNTHETIC MEDIA.

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(*Received August 25th, 1924.*)

In a recent paper [1923, 2] Cutler and Crump have described experiments in which from one to four individuals of *Colpidium colpoda* were isolated into varying volumes of a synthetic culture medium. Previous experiments by the author [1921, 2] had shown that when two individuals of another ciliate infusorian (*Enchelys*) are isolated together into a definite small volume of culture medium, the reproductive rate of both individuals is enhanced so that not merely twice, but from four to sixteen times as many individuals may be produced in 24 or 48 hours as in a culture of the same volume into which only a single individual was originally introduced. It was shown that the effect was not due to conjugation, because conjugation did not occur under the conditions which prevailed in these experiments, while if it had occurred, the result, as Jennings has shown [1913], would have been the opposite of the effect which was observed. This mutual acceleration of reproductive rate by contiguous organisms was termed allelocatalytic effect [1922], and from the autocatalysis which is known to occur during the growth of all organisms, it was inferred that this effect is common to all cells which live in proximity to one another in a limited volume of nutrient medium [1923].

Cutler and Crump, however, failed to observe allelocatalytic effect in their cultures of *Colpidium*. Two possible origins of this divergence of results at once suggested themselves. Either the infusorian employed by Cutler and Crump did not exhibit the effect at all or to a degree much less apparent than *Enchelys*, or else the exhibition of allelocatalytic effect is dependent upon some quality of the nutrient medium which was absent in the synthetic media which were employed by these investigators. The latter alternative appeared the more probable of the two, but in order to exclude definitely the former alternative, it was considered necessary to procure *Colpidium* and repeat the original experiments in hay-infusion medium with this organism and then, with both *Enchelys* and *Colpidium*, to repeat the experiments in the synthetic medium which was employed by Cutler and Crump.

It proved to be a matter of some difficulty to procure *Colpidium* in this locality and much time was consumed in a vain search for this organism. At length it was discovered that a ciliate infusorian closely resembling *Colpidium colpoda*, but considerably larger, is present in small numbers in fresh infusions of various plants obtainable here, but very rapidly disappears as the culture ages, its place being taken by a dense population of *Enchelys* and other ciliates. This *Colpidium* is somewhat longer than *Enchelys*, definitely kidney-shaped and, on the unindented side, it is nearly circular in outline, so that it is almost as broad as it is long, and its volume is therefore considerably in excess of that of *Enchelys*, while the variety of *Colpidium* employed by Cutler and Crump was considerably smaller than *Enchelys* both in length and in breadth and, moreover, was elliptical and not circular in outline. I have been unable to identify this organism more precisely. Unlike *Enchelys* it encysts very readily when deprived of food and from the cysts two or, more frequently, four daughter cells emerge. In the presence of an abundant food-supply, however, it divides, as *Enchelys* does, by transverse binary division.

This organism, *Colpidium* sp., although not identical with the species employed by Cutler and Crump, certainly belongs to the same or a closely allied genus, and it differs sufficiently from *Enchelys*, in morphological detail and physiological behaviour, to afford a satisfactory means of ascertaining the prevalence of allelocatalytic effect among different species of ciliate Infusoria.

The organisms were cultivated in hay-infusion prepared by heating 5 g. of chaffed oaten hay in 100 cc. of distilled water on a boiling water bath for half-an-hour. The filtrate, received into vessels which had previously been sterilised by steaming, was brought to various p_H values by the addition of small quantities of solutions of buffer salts. It was found that *Colpidium* would live and reproduce over a wider range of p_H than *Enchelys*. Satisfactory cultures were obtained in media containing 2 cc. per 100 cc. of $M/15$ phosphate mixture having a p_H of 7·7.

A small number of individuals contained in about 0·003 cc. of culture medium were removed from the parent culture by means of a capillary pipette and placed upon the depression in a slide. To this was added 0·1 cc. of fresh culture medium[1]. From this mixture a single individual was picked out in 0·003 cc. of fluid and isolated into 0·05 cc. of fresh culture medium. This organism was again isolated in 0·003 cc. of fluid and introduced into 0·05 cc. of fresh culture medium, forming the final culture. From the same parent culture two individuals were similarly isolated and re-isolated into a like volume of culture medium. The results were variable in magnitude but invariably of the same character. The reproductive rate of both individuals was much enhanced by their mutual contiguity and instead of twice as many

[1] This was aerated by passage of a stream of air, filtered through sterilised absorbent cotton, for from one-half to one hour before the buffer was added. The boiled hay-infusion does not contain sufficient oxygen for the requirements of the organisms and individuals isolated into unaerated culture media frequently die.

individuals being produced in 48 hours, from four to sixteen times as many individuals were produced in these cultures as in those which originally contained but a single individual.

The experiments were now repeated with one of the synthetic culture-media employed by Cutler and Crump [1923, 1]. This medium had the following composition:

Na_2HPO_4	0·001 %	$MgSO_4$, $7H_2O$ ...	0·0001 %
Ammonium lactate	0·01	$CaCl_2$	0·002
KCl	0·03	Glucose	0·04
NH_4Cl	0·03		

Solutions of the several constituents were prepared which were in each case ten times the final concentration desired. Ten cc. of each solution were then introduced into a 100 cc. graduated flask, a $M/15$ phosphate buffer added in sufficient amount to render the final $p_H = 7·4$ and the volume made up to 100 cc. by the addition of distilled water. The mixture was autoclaved on three successive days and allowed to stand thereafter for several days in a flask plugged with cotton before it was employed. It was not aerated.

Single individuals and pairs of individuals were isolated into this medium in the manner outlined above. The following are illustrative results:

Parent culture	Subculture	No. of individuals isolated into 0·05 cc.	No. of individuals in culture after 24 hours	48 hours
1 A (*Enchelys*)	5 A	1	3	30
1 A ,,	6 A	2	14	120
1 A ,,	7 A	1	3	24
11 A ,,	12 A	1	—	4
11 A ,,	17 A	2	—	11
1 B (*Colpidium*)	15 B	1	1 (dividing)	2
1 B ,,	16 B	2	7 (1 dividing)	18
11 B ,,	17 B	1	1 ,,	—
11 B ,,	20 B	2	4	—

Thus allelocatalytic effect occurs in cultures of *Colpidium* and *Enchelys* in the synthetic culture medium employed by Cutler and Crump, the number arising in 24 or 48 hours in cultures inoculated with two individuals being invariably in excess of twice the number produced in cultures inoculated with a single individual.

The discrepancy between Cutler and Crump's results and my own arises, therefore, neither from the difference of species nor from the nature of the culture medium employed by them. This fact suggested that the discrepancy arose from some detail of experimental technique and a further scrutiny of the methods employed by Cutler and Cramp ultimately revealed its origin.

The accelerative agent which is responsible for the increase of the division-rate of contiguous infusoria in a limited volume of culture medium issues from the cells only during the divisional period. This is shown by the fact that the period of "lag" is in no degree shortened by contiguity of other organisms. I have found, in cultures of *Enchelys* in hay-infusion, that whether one or fifty individuals are isolated into a given volume of culture medium, the period which elapses before the first division occurs, the "lag-period,"

is always the same. It varies with the age of the parent culture, lengthening, as I have previously shown [1921, 1; 1922], as the parent culture increases in density of population, but it does not vary with the number of individuals which are introduced into the subculture. Only after the first division has occurred does the allelocatalytic effect of the contiguous cells become manifest and the effect frequently increases for some time with successive divisions. In a culture containing 65,536 individuals per cc. (16 generations), therefore, the concentration of the catalytic agent will correspond to 65,535 cell divisions, which is the sum of all the divisions which have occurred during the production of 65,536 individuals from a single individual. If a small proportion of this culture be introduced into 100 times its volume of fresh culture medium, the concentration of the accelerative agent in this mixture will be the same as that in a culture in which 655 divisions per cc. have occurred, and if it be introduced into 1000 times its volume of fresh culture medium, the concentration of accelerative agent will still correspond to 65 divisions per cc. Now accumulation of autocatalyst sooner or later leads, as I have shown elsewhere [1922], to *slowing* of the reproductive rate and ultimately reduces it to zero. It is for this reason that the lag-period exhibited by individuals isolated from old parent cultures is so prolonged. In culture media which contain from the beginning such high concentrations of the catalytic agent, therefore, the addition of more by divisions occurring within the fluid, so far from being advantageous, might either leave the reproductive rate unaffected or even retard it. Cutler and Crump inoculated volumes varying from 0·37 to 9·8 mm.^{3} with 0·01 mm.^{3} of fluid obtained from the parent culture. The dilution of the autocatalyst originally present in the parent culture was therefore only from 1 in 37 to 1 in 980, whereas in my experiments, as a simple calculation shows, the dilution of the parent culture fluid is no less than 1 in 9000. After the first division, the concentration of autocatalyst in Cutler and Crump's cultures would rise to that corresponding to 3650 divisions per cc. in the cultures of smaller volume and to that corresponding to 165 divisions per cc. in the cultures of larger volume. In the cultures of smaller volume, therefore, the concentration of autocatalyst would rise very rapidly and soon attain the concentration at which its accelerative effect is replaced by retardation. That the reproductive rate of the organisms was actually retarded by the high concentration of the substances introduced together with the organisms into the subculture may be seen by comparing the average reproductive rates found by Cutler and Crump in their cultures of small and large volume respectively. The following table, compiled from their results, reveals very clearly the retardation which occurred in the culture of smaller volume into which a single individual was introduced:

Volume of subculture in mm.^{3}	Average reproductive rate in 24 hours
0 to 1·5	1·98
1·5 to 3·0	2·27
3·0 to 6·0	2·86
6·0 to 9·0	2·87

In cultures containing two individuals the concentration of autocatalyst would mount still more rapidly and the stage of retardation would be all the sooner attained. So far from obtaining allelocatalytic effect, therefore, either no mutual acceleration of reproductive rate or even retardation might be anticipated, and the results obtained by Cutler and Crump confirm this anticipation.

I have imitated the conditions which prevailed in Cutler and Crump's experiments by omitting the second isolation of the individuals inoculated into the drops forming the subcultures. From a parent culture of *Enchelys* which was four days old and thickly inhabited by infusoria, a small number of individuals were removed in about 0·003 cc. of fluid. This was diluted with 0·1 cc. of synthetic medium and from this mixture one individual was isolated into 0·05 cc. of the synthetic culture medium and two individuals were inoculated into an equal volume of the same medium. This procedure was then repeated, but the individuals now isolated were re-inoculated into 0·05 cc. of synthetic medium, so that while in the first case the substances originally present in the parent culture were only diluted to 1 in 555, in the second case they were diluted to 1 in 9259. The following were the results obtained:

No. of individuals inoculated	Treatment of the individuals inoculated	Individuals produced after 48 hours
1	Not washed by re-isolation	48
2	,, ,,	94
1	Washed by re-isolation	24
2	,, ,,	120

It is evident that allelocatalytic effect was totally abolished by the presence of even 1/555th of the concentration of the substances contained in the parent culture medium but was very marked when their concentration was reduced to 1/9259. The absence of allelocatalysis in Cutler and Crump's cultures, therefore, originated in the contamination of their subcultures by substances derived from the parent cultures. Had the organisms been washed by re-isolation then, as the above results show, allelocatalysis would have been observed[1].

There existed, however, another source of error in Cutler and Crump's experiments which might very readily have obscured the allelocatalytic effect, even if the organisms had been washed by re-isolation. They state that the individual, or individuals, which were inoculated into the subculture were "transferred by a capillary tube from the parent culture with about 0·01 mm.^{3} of liquid." In a previous paper they point out that the distribution of infusoria throughout their cultures was approximately uniform, so that a given fraction of the culture always contained, within narrow limits of error, the same

[1] In a previous paper it is pointed out that washing the infusoria in hyperalkaline distilled water (2 cc. of $N/10$ Na_2CO_3 to 100 cc. distilled water) abolishes the allelocatalytic effect. This is, however, not due, as was then supposed, merely to removal of associated bacteria or their products, but to some specific effect of the hyperalkaline distilled water, for no amount of washing in buffered hay-infusion will abolish the effect.

number of organisms. No details are given of the age or population of the parent cultures from which their subcultures were inoculated, but the data already quoted suffice to show that if in one case 0·01 mm.^{3} of the parent culture contained a single individual, and in another case two, then the population of the latter parent culture must have been greater than (approximately double) the population of the former. Now the lag-period varies with the population of the parent culture, increasing as the population increases, and therefore the lag-period, when two individuals were isolated, must have been longer than in those instances in which a single individual was isolated. The same considerations apply with proportionately greater force to those cultures which contained three or four individuals. If, now, the reproductive rate be estimated from the number of individuals produced in 24 hours, the lag-period will form a large proportion of that time, so that a more prolonged lag-period may offset the effect of an accelerated reproductive rate. Thus, suppose the interdivisional period to be 4 hours in the one case and 2 hours in the other, and the lag-period 16 hours in the former case and 20 hours in the latter. Then in 24 hours the numbers inhabiting the two cultures will be identical, although the reproductive rate in the latter case is actually double that obtaining in the former. This source of error was avoided in my experiments because the single and the paired individuals were isolated from the same parent culture and, as indicated above, the lag-period does not vary with the density of the initial population of the subculture. The number of individuals present after 24 hours must, in this event, be greater in the culture having the higher reproductive rate[1].

Cutler and Crump have repeated and confirmed my observation that cultures of infusoria contain a substance or substances capable of accelerating the multiplication of infusoria in scantily populated cultures, and they also show that similar accelerative substances may be extracted from the crushed bodies of infusoria. They find that the acceleration due to the addition of these substances to the culture medium is only exhibited after some delay and they state that "This is contrary to the findings of Robertson and is difficult to understand on the analogy of enzyme action." These authors are, however, labouring under a complete misapprehension of the facts, and it appears necessary to quote, therefore, the following remarks from my paper

[1] It was, however, for this reason that I was formerly led to report that allelocatalytic effect is diminished or absent when individuals from old parent cultures are inoculated together into the same drop [1921, 2]. Not only must the washing of individuals taken from old parent cultures be more thorough to remove the accumulated autocatalyst, but also allowance must be made for the fact that proportionately slight variations in the length of the lag-period will lead to great apparent differences of reproductive rate, if the lag period is long and included in the estimation of division-time. Obviously the effect of variations in lag, if they are not too great, will be minimised as multiplication proceeds and hence allelocatalytic effect which is not apparent in 24 hours frequently becomes quite evident in 48 hours. If these sources of error are carefully excluded, allelocatalytic effect is just as apparent in cultures inoculated with individuals derived from old parent cultures as it is in cultures inoculated with individuals derived from young parent cultures.

on "Reproduction in Cell-communities" [1922]: "Now I have repeatedly observed that the effect of accelerative agents upon the multiplication of infusoria is not manifested to any important degree during the period of lag....The accelerative agent which is discharged by multiplying infusoria into buffered distilled water does not abbreviate the lag-period of infusoria isolated into nutrient media which contain it. Again, yeast extract shortens the lag-period only to a relatively insignificant degree while, subsequently to the first division, the reproductive rate is decidedly enhanced." Since the greater part of the first 24 hours after isolation of infusoria from a densely populated parent culture is consumed by the lag-period, it will be clear that Cutler and Crump's results are, in this particular, absolutely concordant with mine. Nor are they at all difficult to understand if one accepts their obvious meaning, that the accelerative agents concerned act upon the nucleus and cannot enter or leave the nucleus, except during the divisional period when the nuclear membrane no longer interposes a barrier between the nucleus and the cytoplasm which envelops it.

Greenleaf [1924], working with *Paramoecium* and *Pleurotricha*, has also recently reported failure to observe allelocatalytic effect in cultures of these organisms. While his account of his experimental technique is very meagre the origin of the negative results obtained in these experiments seems to be sufficiently obvious. He isolated single individuals into volumes of hay-infusion varying from two to forty drops and compared the populations attained after five days, from which the rate of multiplication per diem was computed. Now the maximal density of population in an infusorial culture does not vary greatly with the volume of the culture medium, provided, that is, that the infusoria originally inoculated into the culture are adequately washed to remove autocatalyst derived from the parent culture. Hence in twenty drops, for example, more divisions would be required to attain maximal population than in two drops. A simple calculation shows that if forty drops = 1 cc., then a maximal population of 65,000 individuals per cc. will arise from a single individual in from eleven to twelve generations in two drops of culture fluid, and not until sixteen generations in forty drops of culture fluid. In cultures of *Enchelys* and *Colpidium*, the maximal population of one or even two drops of culture medium originally inoculated with a single individual may be attained within three days if the lag-period is not extraordinarily prolonged. Hence in Greenleaf's experiments the cultures in the small volumes had ceased to reproduce some time before the count was made, while those in twenty drops may have been still dividing[1], and of course the rate per diem calculated from these populations appeared higher for the cultures of larger volume. In my experiments on the influence of culture volume upon the reproductive rate of isolated *Enchelys*, the initial or early rates of reproduction were compared and found to stand in nearly inverse proportion to the volume

[1] For, as Cutler and Crump have shown, the interdivisional periods are much more prolonged as the population approaches a maximum.

of the culture. It did not at that time appear to me to be necessary to point out that the superiority of reproductive rate in cultures of small volume could not possibly continue after, or even near, the attainment of maximal population. Greenleaf also failed to obtain allelocatalytic effect in cultures of equal volume containing one and two individuals respectively. Again, it appears necessary to draw attention to the obvious fact that two individuals isolated into a small volume of culture medium will produce a maximal population more quickly than one, and their progeny will, in consequence, cease multiplying while the progeny of the single individual are still undergoing reproduction.

SUMMARY.

1. Cutler and Crump failed to observe allelocatalytic effect (the mutual acceleration of reproductive rate by contiguous organisms) in cultures of *Colpidium colpoda* in a synthetic medium.

2. The author has observed allelocatalytic effect in cultures of *Colpidium* sp. and *Enchelys* in hay-infusions and also in the synthetic medium employed by Cutler and Crump.

3. The failure of Cutler and Crump to observe allelocatalytic effect was due to the omission of the precaution of washing the organisms by re-isolation from a relatively large volume of culture medium before inoculation. It is shown that the dilution of the substances contained in the parent medium was only from 1 in 37 to 1 in 980 in the cultures employed by Cutler and Crump, whereas in the author's cultures it was 1 in 9000. It is shown that the concentration of parent culture medium contained in Cutler and Crump's cultures was sufficient to suppress the allelocatalytic effect and the cause of this suppression is explained.

4. In comparing reproductive rates in infusorial cultures the individuals inoculated into the cultures must all be derived from one parent culture, so that the lag-period is the same for all of them, or else the variable period of lag must be subtracted from the total period occupied in multiplication before the reproductive rates are compared.

5. In comparing reproductive rates in infusorial cultures care must be taken to estimate the population some time before it has attained its maximal density, for whatever the speed with which maximal population may be attained its final density in any given culture medium and at any given temperature is approximately the same.

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CLXIII. THE EFFECT OF FERMENTATION ON THE WATER-SOLUBLE VITAMIN CONTENT OF WORT.

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(*Received September 29th, 1924.*)

In a previous communication [1924] the writer has shown that there is much less water-soluble B vitamin in beer than in the corresponding amount of malt used in its manufacture. In this connection it should be stated that Harden and Zilva [1924] in their investigation of the subject, report the beer samples tested by them to be free from water-soluble B vitamin. It was indicated that further experiments were in progress to determine if possible where the main loss occurred in this process. The prevalent idea appears to be that beer is indebted to the yeast cell for its content of this factor. The correctness of this theory is dependent on the answer to the question whether yeast does or does not synthesise the water-soluble B factor during the fermentation of the wort. The results obtained by Eijkman and his co-workers [1922] showed that during the process of fermentation of wort by the yeast cell the water-soluble factor passes from the wort into the yeast cell. The quantitative experiments described below corroborate their findings and in addition show that there is no actual synthesis of this factor during the process.

The experiments were as follows:

To 1200 g. of coarsely ground malt were added 3 litres of water previously heated to 150° F., and the mixture was maintained at this temperature for two hours. At the end of this period the wort was filtered through glass wool and the grains repeatedly washed with water at 150° F. till another 3 litres had been used. The filtrates were mixed, this wort having a gravity of about 1057 and a volume of roughly 5 litres. This was then brought to the boil and then autoclaved for 20 minutes at 125° C. The precipitate which had formed during this last process was removed by filtration through sterile glass wool from the dark coloured wort which was equally divided into two sterile 3 litre flasks. Each flask thus contained roughly 2½ litres of wort. For the fermentation a small amount of top yeast was obtained from a local brewery, plated out on wort-gelatin and colonies morphologically resembling *S. cerevisiae* were sub-cultured into sterile wort of similar gravity to the above, and after vigorous growth, again sub-cultured and grown in 15 cc. lots of sterile wort in test-tubes. After growing for three days at a temperature of 69° F., each tube yielded about 1 g. of moist yeast, on centrifuging down the liquid for ten minutes

at 3000 R.P.M. Two of these tubes of fermented wort were taken, the contents of one added to those of one of the 3 litre flasks, and fermentation allowed to proceed at 69° F. for three days, after which time the gravity had dropped to 1012–1014. The contents of the other tube were just brought to the boil and then added to the second 2500 cc. of sterile wort to compensate.

When active fermentation had ceased in the first flask the beer was cleared of yeast cells by centrifuging. The weight of moist yeast obtained was 50–55 g. Thus about a fifty-fold multiplication had occurred. The volume of the beer was about 2200 cc. The yeast was suspended in water and alcohol added to make the strength of the latter 20 %, when the total volume of the yeast suspension was 440 cc. Thus 440 cc. of this are equivalent to 2200 cc. of beer. The yeast suspension was kept in the ice-box and always well shaken just before use. The beer was brought to the boil and again autoclaved. The other $2\frac{1}{2}$ litres of wort were subjected to the same process to compensate for any loss of vitamin through heating. Each of the 3 litre flasks was fitted with sterile tubes and covered delivery pipettes for syphoning over the liquid. The sterility of the beer and wort was thus maintained.

The precipitate was suspended in aqueous alcohol (4: 1), and the volume made up 440 cc. This was also kept in the ice-box.

The four test substances were then examined for their content of water-soluble growth-promoting factor on rats quantitatively, by adding to the basal diet (see previous paper), which was deficient in water-soluble B vitamin, quantities of 10 cc. of wort, 10 cc. of beer, 0·5 cc. of yeast suspension and 1 cc. of precipitate respectively; these amounts being increased to 15 cc., 15 cc., 0·75 cc. and 1·5 cc. respectively.

The result illustrated in Fig. 1, with rats of the same family, has been obtained with five other sets.

Fig. 2 shows the result of an experiment to test whether there was any actual synthesis of the water-soluble growth promoting factor by the yeast cell growing under the above conditions. In this case the yeast cells were not centrifuged down after fermentation, but the flask contents just brought to the boil and then rapidly cooled. 10 cc. increasing to 15 cc. of wort and an equal volume of the beer-yeast-mixture were then fed to rats with the results shown. It should be mentioned that the wort was similarly brought to the boil and cooled, in order to compensate.

A consideration of the above results shows, first, that the yeast has abstracted the growth promoting factor almost quantitatively from the wort. The presence of vitamin B in the beers of commerce as described in the author's previous paper is probably due to the fact that wort in the vat is much more heavily seeded with yeast for fermentation. Since it has been shown that within certain limits the number of yeast cells at the end of the fermentation process is independent of the number used for seeding, the multiplication will vary inversely with the quantity used in seeding. I am informed that about one pound of yeast is used per barrel and at the end of fermentation this has

increased to six pounds—a much smaller multiplication than was obtained in the experiments described above, in which the increase was fiftyfold.

Secondly it is evident that there has been no synthesis of the factor by the yeast cell during the process, but rather a small fraction of the factor originally present in the wort has disappeared. It would be rash to conclude from these experiments that the yeast during this process has actually used up some of the growth-promoting factor since the difference is small, but the results suggest this.

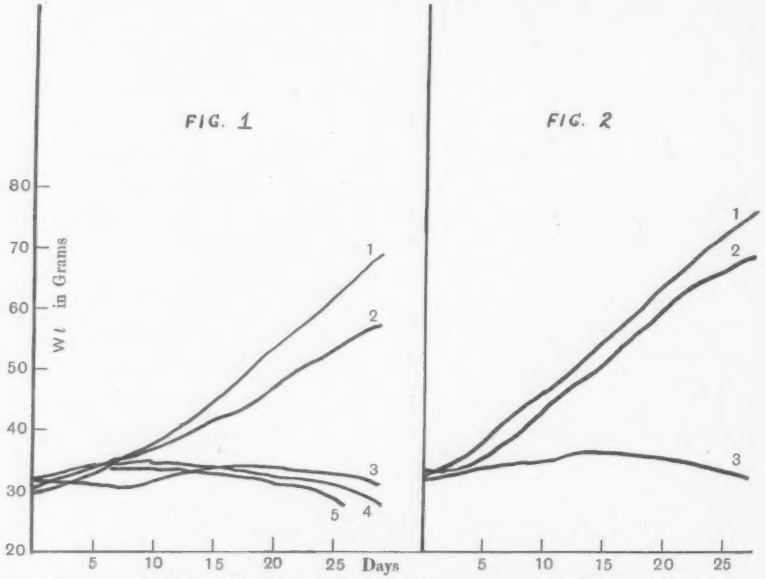

Fig. 1. Curve 1, 10–15 cc. of wort added to the basal diet of this rat. Curve 2, 0·5–0·75 cc. of yeast suspension added to the basal diet. Curve 3, 10–15 cc. of beer added to the basal diet. Curve 4, rat on basal diet only. Curve 5, 1–1·5 cc. of the precipitate suspension added to the basal diet. All these rats were females.

Fig. 2. Curve 1, 10–15 cc. of wort added to the basal diet of this rat. Curve 2, 10–15 cc. of the beer yeast suspension added to the basal diet. Curve 3, rat on basal diet only. The three rats were males.

Thirdly, the experiments described above, and others in which larger quantities of the precipitate were used, show that the precipitate from the first autoclaving did not carry down any measurable quantity of the factor.

The relationships of the water-soluble B vitamin to the yeast cell have long been the subject of experimental investigation—and controversy. Some of the aspects of the problem have been discussed by Heller [1922] and others. The difficulties have been increased rather than diminished by the work of Funk and Dubin [1921] corroborated by Heaton [1922], and more recently by Deas [1924]. From the work of these authors there can no longer be any

doubt that bios is distinct from vitamin B. Whether vitamin B is essential for the growth and multiplication of the yeast cells the above results do not prove, but suggest it. Clark [1922] showed that bios is rapidly and completely taken up from wort when the latter is shaken up with yeast, even when a large amount of yeast is used with no multiplication taking place. Eijkman showed that under similar conditions the antineuritic factor is not removed. This contrast has been pointed out, not so much with the idea of drawing another distinction between bios and the water-soluble B factor, as suggesting the possibility that vitamin B is taken up by the yeast cell during its growth and multiplication.

Summary.

1. When the yeast cell is allowed to grow in beer wort—its optimum medium—it abstracts almost quantitatively the water-soluble growth-promoting factor from the medium, when grown under the above conditions.

2. The organism does not synthesise any of this factor during this process, but if anything actually uses up some of that which it takes up from the wort.

3. The main loss of vitamin B in the preparation of beer is thus accounted for.

Finally it may be added that the question of the loss of the growth-promoting factor during the malt-wort transition is at present being investigated.

In conclusion I would express my indebtedness to Professor Edward Mellanby for his help throughout the work. The expenses of this research have been defrayed by the True Temperance Research Committee.

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CLXIV. GLYCURONIC ACID AS A PROBABLE CONSTITUENT OF UROCHROME.

(PRELIMINARY COMMUNICATION.)

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(*Received September 22nd, 1924.*)

Urochrome has been carefully prepared from human urine, in a state believed to be pure, by a method combining and modifying in some details the methods of Garrod and Weiss. The product when boiled with 10 % HCl yields vapours, which give a crimson colour with aniline acetate and therefore probably contain a furfural. Even in small traces it gives a well-marked Tollens-Neuberg reaction and also the Van der Haar [1918] modification which is claimed to be specific for glycuronic acid. In this latter test, the substance investigated is heated for one minute in a boiling water-bath with 10 cc. of a mixture of water and one-third its volume of strong hydrochloric acid, and at least 100 mg. of naphthoresorcinol, then cooled to 50° and shaken up with benzene. The separated layer of benzene is dried by shaking with anhydrous sodium sulphate and filtered, and then has a violet colour and an absorption band on the D-line. No other carbohydrate or derivative gives this test.

The above facts make it highly probable that urochrome is a glycuronic acid compound, and the question is under further investigation.

My thanks are due to Professor Ramsden for suggesting the work.

REFERENCE.

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CLXV. THE ISOLATION OF SPERMINE PHOSPHATE FROM SEMEN AND TESTIS.

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(*Received October 3rd, 1924.*)

In the course of experimental work on "sperma crystals," extending over the last twenty years, I made an exhaustive study of the literature, as an outcome of which a new chapter must be added to the already remarkable history of an equally remarkable substance. According to the statements of the text-books, accepted by all writers on the subject, these peculiar crystals were discovered by Böttcher [1865] in human semen and they have since been known as "Böttcher's crystals." Long before Böttcher, however, they were accurately described by Vauquelin [1791], and even this distinguished observer only rediscovered the crystals which, more than a hundred years before, had been seen and depicted by Leeuwenhoek [1678]. The passage describing the crystals occurs at the end of the famous communication to the Royal Society in which Leeuwenhoek announces the discovery of spermatozoa. The rough marginal sketch (see Fig. 1), like many others drawn by Leeuwenhoek in his letters to the Royal Society, has suffered somewhat in reproduction by the hand of the engraver, but in the following few words, he gives an admirable description of the "glittering, translucent" crystals, including even their correct measurement[1]: "Et cum praedicta materia paucillum temporis steterat, in ea observabantur trilaterales figurae ab utraque parte in aculeum desinentes, quibusdam longitudo minutissimae arenae, aliquae aliquantulum majores ut fig. A. Praeterea, adeo nitidae ac pellucidae, ac si crystallinae fuissent."

Fig. 1

Vauquelin [1791], evidently unaware of Leeuwenhoek's previous observation, characterised the crystals as possessing "la forme d'une solide à quatre pans, terminés par des pyramides très allongées, a quatre faces." He was the first to attempt their chemical investigation, and the analysis of an obviously impure preparation led him to the diffidently expressed view that they repre-

[1] Leeuwenhoek's "smallest grain of sand" measures according to his own scale approximately one-hundredth of an inch, *i.e.* 0·25 mm. For this and other information regarding Leeuwenhoek's work, I am much indebted to Mr Clifford Dobell, who also kindly lent a copy in his possession for the reproduction of the above-quoted passage.

sented a hitherto unknown crystallised form of calcium phosphate. Subsequently Berzelius [1833] mentions the crystals in his investigation of semen and, without attempting their analysis, suggests that they consist of ordinary ammonium magnesium phosphate crystals, as so frequently found in other animal fluids[1]. Probably on Berzelius' authority the text-book writers of the period accepted this view, and the crystals are thus fully described by C. G. Lehmann [1853]. Their existence might therefore have been known to Böttcher [1865], when he "rediscovered" and described the crystals under the title "Farblose Krystalle eines eiweissartigen Körpers aus dem menschlichen Sperma." Böttcher's unconvincing evidence for the protein nature of the crystals, based on microchemical reactions, was accepted by subsequent text-book writers, as for instance by Kühne, who assumes them to be vitellin, and by Hoppe-Seyler who compares them to aleurone grains of seeds.

Ph. Schreiner [1878] was the first to recognise the true nature of the crystals. In a classical paper, which unfortunately lacks essential experimental details, Schreiner came to the conclusion that the crystals were the phosphate of a new organic base. The material obtained by him from semen was evidently just enough for an analysis and a study of the properties of the base. With this knowledge he succeeded in demonstrating that a base, yielding the same characteristic insoluble phosphate, is widely distributed in the animal organism. From the analysis of the hydrochloride he deduced the formula C_2H_5N, a formula with which his analytical figures for the phosphate are not in agreement, as pointed out by Ladenburg and Abel [1888]. It will be shown later [see Dudley, M. C. Rosenheim and O. Rosenheim, 1924] that Schreiner's analysis of the hydrochloride is faulty, whilst his figures for the phosphate correspond to the correct empirical composition of the base.

Schreiner did not name the new substance, and it was known as "Schreiner's base" or "spermatin" for some time [see Thudichum, 1879; Fürbringer, 1881]. The name "spermatine" had been previously used by Berzelius [1833] for a specific protein of semen, and the name "spermin," apparently first applied to the base by Ladenburg and Abel [1888] has since been in use.

It is a remarkable fact that no investigator since Schreiner has again been able to isolate spermine phosphate from semen, so that quite recently Wrede and Banik [1923], after an unsuccessful attempt, consider it doubtful whether the phosphate will ever be obtained again. Nor have the attempts to verify Schreiner's statements of the occurrence of spermine in testis been successful [Majert and Schmidt, 1890; Merck's Chem. Works, Parke Davis & Co., see Jürgens, 1890]. Its existence in animal organs has even been doubted by Wrede and Banik, who were evidently unaware of the recent confirmatory work of M. C. Rosenheim [1917][2].

[1] This view is still held by at least one modern worker, who also denies the existence of spermine in semen [Slowtzoff, 1902, 1916].

[2] Since it has now been established by M. C. Rosenheim [1917] that spermine passes into the lysine fraction, it is interesting to find that various authors came very near to its rediscovery in spleen and pancreas. Gulewitsch and Jochelsohn [1900], examining the lysine fraction of an

The only author who claims to have isolated spermine phosphate from testis and other organs is the Russian physiologist, A. von Poehl [1891]. His chemical investigation, incomplete and vague as to details, stands discredited together with his pharmacological and clinical work, on account of his persistent commercial exploitation of the assumed valuable therapeutic properties of spermine [see Poehl's "Spermin Theorie," 1898, .1906]. Although the commercial material, "Sperminum Poehl," of which I examined a sample recently, contained only traces of spermine, there is no doubt that Poehl had actually isolated spermine phosphate and that his single analysis of the chloroplatinate, to which he ascribed the formula $C_5H_{14}N_2 . H_2PtCl_6$, is correct. Through the kindness of Prof. Halliburton, I came many years ago into the possession of a specimen of spermine phosphate which had been prepared and presented to him by the late Prof. Poehl. Crystallographically and chemically Poehl's specimen is identical with the product prepared by me from semen and testis.

In my own earlier attempts to prepare spermine phosphate from semen, I invariably failed, as no doubt other observers before, and Wrede since has done, to isolate the crystals. This failure was not due to the absence of the substance, but is inherent in the method as described by Schreiner, as will be shown in the experimental part. I succeeded finally by adopting a slight, but essential, modification, suggested by the experience with Vauquelin's original sedimentation process. The crystals can also be obtained directly from semen by dialysis. During the progress of the work on the isolation of spermine from animal organs which is described in the subsequent paper [Dudley, Rosenheim and Rosenheim, 1924], the observation was made that the free base is easily extractable from alkaline solution by butyl alcohol, and further that it can be distilled with steam. Methods of isolation from semen based on these observations will be described.

It will further be shown that the presence in testis of spermine phosphate as such can easily be demonstrated. The identity of the various preparations from semen and testis with those from pancreas, spleen, yeast, etc., has been proved by their crystallographic analysis, by their melting points, and by the preparation from them of the picrate, picrolonate, chloroplatinate and chloroaurate of spermine.

Experimental.

Leeuwenhoek's discovery may be easily verified and the striking phenomenon of the appearance of the characteristic crystals can best be followed under the microscope by mounting a drop of fresh semen on a slide under a

extract of ox spleen, obtained 20 mg. of a base which gave a microcrystalline precipitate with phosphoric acid, the identity of which with spermine they were unable to decide owing to lack of material. Kutscher and Lohmann [1904, 1905] state that they repeatedly obtained from the lysine fraction of autolysed pancreas the picrolonate of an unidentified base, which according to their description and nitrogen determination may now probably be identified with spermine picrolonate. Werigo's [1892] "cadaverine picrate" from pancreas was very likely spermine picrate.

coverslip. The shortest time of their appearance is, according to my observations, from forty to sixty minutes, after the semen has undergone the peculiar rapid change from the viscid tenacious condition into a thin watery fluid. Since spermine phosphate as such exists in testis and other organs, its crystallisation from semen is most easily explained by changes in temperature and hydrogen ion concentration.

With regard to their size, my observations agree with those of previous observers. Their average length is 0·25 mm., but crystals up to 2 mm. in length may occasionally be seen.

(1) *Isolation from semen.*

It may safely be said that it was not the difficulty of procuring material alone, but rather the uncritical adherence to Schreiner's directions which caused the failure of subsequent observers to isolate the crystals, and thus to doubt their existence [see Wrede and Banik, 1923]. It is unfortunate that Schreiner, in ignorance of the simple procedure adopted by Vauquelin, chose a method which must necessarily lead to failure when carried out exactly as described by him. As the reasons for this statement became apparent only after the successful isolation of the crystals by other methods, it will be advisable to follow the historical sequence.

(*a*) *Modification of Vauquelin's method.* Vauquelin observed that the crystals, being heavier than the fluid in which they are suspended, fall to the bottom when fresh semen is allowed to stand for some time in a narrow vessel. He decanted the supernatant fluid and washed the crystals by decantation with cold water, in which they are insoluble. By this simple process he succeeded unknowingly in removing the peculiar protein of semen, which prevents the isolation of the crystals by Schreiner's method. On the other hand, this crude method must necessarily yield a very impure preparation, since the sediment is seen, on microscopical examination, to consist of a mass of crystals entangled with spermatozoa and débris. By adopting, however, the principle of Vauquelin's method, the crystals can be easily isolated in a fairly pure condition.

Fresh semen is allowed to stand in a centrifuge tube for some hours. The contents of the tube are then well mixed with an equal volume of 0·9 % sodium chloride solution, and the sediment centrifuged off. It is freed from soluble proteins by repeatedly washing it in the centrifuge with salt solution, subsequently with water and with alcohol. It is now boiled up with alcohol, centrifuged when cold, and finally washed with ether. A white powder remains which can be easily removed from the tube and extracted with boiling water. A perfectly clear filtrate, free from proteins, results, from which the crystals separate on cooling, if necessary after suitable concentration and the addition of a few drops of alcohol. Yield from 4·5 cc. semen: 6 mg. = 0·14 %. From 25 cc. semen, collected and worked up in portions of about 5 cc., were obtained 32 mg. spermine phosphate = 0·13 %.

(*b*) *Modification of Schreiner's method.* The method as described by Schreiner is shortly as follows: "Fresh semen is boiled with alcohol in order to coagulate the proteins. After some hours the precipitate is filtered off and dried. The substance is finely ground and extracted with warm water, to which a few drops of ammonia have been added. Only traces of proteins pass into solution, whilst the phosphate is completely extracted and crystallises out in its peculiar forms on concentration of the extract. In a quantitative experiment the dry residue of semen yielded 5·2 % of the crystals."

There is no difficulty in repeating the first part of Schreiner's directions, which are only a confirmation of Vauquelin's and Berzelius' previous observations. Semen is precipitated by alcohol as a perfectly white stringy mass, which, when filtered and dried, amounts to about 10 % of the semen. When this precipitate, however, is ground up with water it swells up and forms a colloidal solution. Contrary to Schreiner's statement, it is found that on warming or boiling the gelatinous fluid practically the whole of the protein goes into solution. Filtration through paper, with or without pressure, soon stops, and as a rule only a few cubic centimetres of filtrate are obtained. On slow evaporation of the filtrate transparent membranes form on the surface of the fluid, drying to varnish-like films in which well-formed crystals of spermine phosphate are embedded.

The following slight, but essential, modification of Schreiner's method led to the isolation of the crystals in a fairly pure condition. The dried alcohol precipitate is finely ground and treated with water at room temperature for a few hours until a homogeneous suspension results. On centrifuging, a colourless protein solution and a white sediment are obtained. The solution is decanted and the treatment with cold water repeated until the supernatant fluid no longer yields a precipitate when poured into five volumes of alcohol. Success depends on the efficient removal of the water-soluble protein, and at least five extractions are necessary. The sediment is then washed in the centrifuge with cold alcohol, afterwards with boiling alcohol, and finally with ether. There remains a white powder amounting to 30–40 % of the weight of the original substance. On extraction with boiling water, this powder gives a water-clear filtrate, free from protein, from which the characteristically curved spindle-shaped crystals of spermine phosphate deposit on cooling. The yield is approximately the same as that obtained by the previous method. From 14·5 cc. semen, 25 mg. of the phosphate = 0·17 % were obtained.

It is evident from these experiments that human semen contains a peculiar protein soluble in cold water and not coagulated on boiling. By pouring the watery extracts, obtained at room temperature as above, into five volumes of absolute alcohol a voluminous white precipitate was obtained, which was centrifuged off, and washed with alcohol and ether. On drying it shrank to a horny mass which could be easily powdered. The fine white powder is easily soluble in water, reacting strongly alkaline to litmus. The solution is not coagulated by boiling, even when acidified with acetic acid. It gives the

biuret reaction and is precipitated by picric acid as well as by other alkaloidal reagents, in acid and alkaline solution. In several quantitative experiments its amount varied from 35–40 % of the original dry semen. According to its reactions, this protein belongs to the group of protamines which have been isolated by Kossel and his co-workers from the spermatozoa of fishes. The existence of this substance as a specific constituent of human semen had already been noticed by Berzelius [1833], who called it "spermatine." More recently Posner, and especially Slowtzoff [1902, 1916] took the substance for a "propeptone" or albumose. Hofmann [1909] isolated by Kossel's method a small amount (0·36 g.) of a substance from human semen, which on the basis of somewhat scanty evidence, he considers to be protamine sulphate.

Schreiner was clearly unaware of the existence of this protein, and it is difficult to understand from his description how he avoided its interference with the isolation of the crystals. Perhaps the surprisingly large yield of spermine phosphate (5 % of the dry semen), which he claimed to have obtained, was due to the admixture of protamine.

(*c*) *Dialysis.* Although pure spermine phosphate is only slightly soluble in water, it is possible to separate it from semen by dialysis. For this purpose it is advisable to use "dry semen" prepared as above by precipitation with alcohol, since choline and other dialysable substances are thus removed. The powder is boiled with about ten volumes of water, in order to ensure sterilisation and to bring the phosphate into solution. A membrane condom (sheep's caecum) was used as a dialyser, and toluene as an antiseptic. Dialysis is very slow, and had to be continued for eight days, the dialysate being removed every 24 hours. After concentration, a small amorphous precipitate was removed by filtration, and crystallisation started by the addition of a few drops of alcohol. Particularly well-formed crystals of spermine phosphate were obtained. The alcohol-precipitate from 90 cc. semen yielded 0·20 g. = 0·22 %.

(*d*) *Butyl alcohol method.* 50 cc. semen were precipitated with alcohol as described above. The dry powder was boiled up with 70 cc. water, faintly acidified with acetic acid, and precipitated with 7 cc. of a saturated lead acetate solution. The precipitate settled on slight warming, and was centrifuged off. The opalescent solution, which gave no further precipitate with basic lead acetate, was freed from lead with sulphuric acid and filtered through kieselguhr. The clear filtrate was made strongly alkaline and extracted with butyl alcohol in an apparatus for continuous extraction, made entirely of glass. Four extractions, lasting six to eight hours each, were made. The colourless extracts were precipitated with a 25 % solution of phosphotungstic acid in butyl alcohol. The white precipitate was allowed to settle and washed four times by decantation with acetone. After filtration and drying *in vacuo*, the phosphotungstate was obtained as a white voluminous powder, weighing 3·6 g. This was decomposed by baryta in the cold, filtered and freed from excess of baryta by CO_2. The final filtrate measured 30 cc. One-third of it was acidified

with dilute phosphoric acid to Congo red, and the remaining two-thirds added. The resulting solution reacts amphoteric to sensitive litmus paper, and deposits well-formed crystals of spermine phosphate on standing. The crystallisation was hastened and completed by the addition of 10 cc. alcohol. Yield 0·12 g. = 0·24 %.

(*e*) *Steam distillation.* The alcohol precipitate from 5 cc. of semen was prepared as previously described, dissolved in 10 cc. of strong alkali and steam distilled in a Claisen flask. Distillation was continued until a drop of the distillate no longer reacted with potassium bismuth iodide or picric acid. (The picrate from another experiment had the correct melting point of spermine picrate.) Altogether 300 cc. of the distillate were collected and evaporated nearly to dryness. The residue was dissolved in 1 cc. water, and 0·5 cc. of a 20 % solution of ammonium phosphate was added. Crystallisation began immediately on scratching with a glass rod, the phosphate depositing as a heavy white powder. The phosphate may also be prepared by neutralisation of the distillate with phosphoric acid, as described under (*d*). Yield 14 mg. = 0·28 %.

As regards the choice of methods, the steam distillation is the most rapid and satisfactory as to quality and quantity of product. The purely physical methods described under (*a*), (*b*), and (*c*) are tedious and yield on the whole a somewhat less pure product. The crystals obtained by these methods usually show besides the characteristic spindle-shaped curved crystals, a certain proportion of prismatic crystals, as frequently seen in sperma itself. This tendency persists on repeated recrystallisation. Fig. 2 (Plate VIII) shows typical crystals of spermine phosphate prepared from semen, testis, pancreas and yeast.

(2) *Spermine phosphate in testis.*

The existence of spermine in testis has been definitely denied by Majert and Schmidt [1890] in connection with their work on piperazine and its supposed identity with spermine[1]. They worked up fifty bull's testes by Schreiner's method without obtaining a trace of the base. Their failure is easily explained by their unfamiliarity with the peculiar properties of spermine phosphate and the difficulty of obtaining it crystallised except under certain well-defined conditions.

The presence of spermine phosphate in testis can be easily demonstrated when the tissue is "plasmolysed" by means of ether vapour. A highly concentrated "exudate" of the water-soluble cell contents is thus obtained, from which spermine phosphate and proteins are precipitated by alcohol. Their separation is carried out as previously described. The method is too tedious for the isolation on a large scale, especially as the yield is only 10–15 mg. from six testes (2 kg.).

[1] See Poehl [1898] for details of this unfortunate episode in the spermine history which caused much confusion, but is now only of secondary interest.

Böttcher had already noticed crystals resembling spermine phosphate on the surface of old pathological-anatomical preparations, and this observation was confirmed by Schreiner[1]. I repeated this experiment by suspending a bull's testicle, weighing 340 g., in 700 cc. 96 % alcohol in a stoppered bottle. Three weeks later crystals of spermine phosphate, singly and arranged in rosettes, could be seen under the microscope in the scummy deposit from the surface of the organ. After three months macroscopic crystals of 3–4 mm. in length had formed. The crystals were scraped off from the tissue and the sides of the bottle, boiled up with alcohol and the residue extracted with water. Typical crystals of spermine phosphate, weighing 6 mg. = 0·002 % were obtained. The yield is only one-third of that obtained by the methods to be described later.

It seems justifiable to conclude from these experiments that spermine phosphate exists as such in the organ, unless we assume it to be formed post mortem by enzymic processes in spite of the presence of ether and alcohol.

The identity of the spermine phosphate preparations from semen and various animal organs, etc., has so far not been proved by strictly chemical methods. Schreiner unfortunately failed to give its melting point; he merely mentions its behaviour when heated on a platinum foil and says that the crystals "liefern bei circa 170° C. eine etwas gebräunte, beim Erkalten wieder fest werdende Schmelze." This observation forms the basis of the statement, transmitted in successive generations of text-books, that the melting point of spermine phosphate is 170°. In his monograph, Poehl [1898] gives it at 130–135°, presumably for a testis preparation. In one of his earlier communications [1892], however, occurs the statement that the dry substance, prepared from pancreas, melts between 230–240°.

The melting points of spermine phosphate, prepared from semen, and of two other preparations, from testis and yeast respectively, were taken simultaneously on the same thermometer. The specimens showed identical behaviour, frothing up and melting between 230–235° (uncorr.), finally running down the capillary and solidifying to a white amorphous mass on cooling. A mixture of the phosphates from semen and pancreas showed no depression of the melting point.

On heating in a test-tube all the preparations give a strong pyrrole reaction with a strip of pine wood dipped in hydrochloric acid. They all show the ninhydrin reaction [M. C. Rosenheim, 1917].

One of the most characteristic salts of spermine is the picrate. Like the phosphate, it is extremely insoluble in cold water, and crystals of more than 1 cm. in length were obtained from the phosphates prepared from semen by any of the methods described above. It melts at 249–250° with frothing. A mixture with the picrate from pancreas showed no depression of the melting point. The crystallographical examination of spermine picrate proves inci-

[1] Böttcher's statement that the crystals forming in egg-white on standing are identical with spermine phosphate was not confirmed by M. C. Rosenheim [1917].

dentally that this is the substance responsible for Barberio's semen reaction with picric acid, a reaction of diagnostic value in forensic medicine, which has been widely discussed without receiving a conclusive explanation. (For literature see Littlejohn and Pirie [1908], and especially B. Baecchi [1912].)

I further prepared the picrolonate (M.P. 288–289°), the chloroplatinate (M.P. 245°), and the chloroaurate (M.P. 225°)[1]. Their melting points are identical with those of the salts prepared from pancreas and other organs to be described fully in the joint communication with H. W. Dudley and M. C. Rosenheim.

Mr T. V. Barker, of the Mineralogical Department, University of Oxford, has kindly carried out the crystallographic examination of the various salts.

Report by T. V. Barker.

The colourless crystals of spermine phosphate obtained from semen are identical in all their geometrical and optical characters with those derived from pancreas. The form of the crystals is generally lenticular. In absence of any straight edges the long axis of the crystals was selected as a line of reference, and gave an extinction angle of 22° between crossed Nicols. The refractive index of the ray making the smaller angle with the crystal axis is 1·50, whilst the other ray gave a value 1·53. The specific gravity is 1·42.

The picrates also gave identical constants. The crystals are yellow flattened prisms with straight extinction. The refractive index of the ray vibrating along the prism has a value 1·503. The other ray has a much higher value (possibly in the neighbourhood of 1·9), and could not be determined by immersion methods.

The chloroplatinates were obtained in measurable crystals, and thus allowed a most rigorous proof of identity. The salt of pancreatic origin crystallises in brilliant-red monoclinic prisms of the habit shown in Fig. 3, the forms developed being m (110), r (101), q (011) and s ($\bar{2}31$). The elements $a : b : c = 0{\cdot}5672 : 1 : 0{\cdot}7840$, $\beta = 116° 44'$ were computed from the following mean values derived from two crystals:

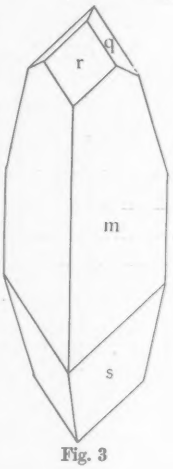

Fig. 3

	m	r	q	s
ϕ	*63° 8′	90° 0′	*32° 44′	312° 17′ (0)
ρ	90 0	63 57 (2)	*43 0	74 3 (− 1)

In polarised light the extinction on m makes an angle of 22° with the prism edge. An optic axis is visible through m on the edge of the field.

[1] In a recent paper Wrede [1924] describes the chloroaurate (M.P. 216–218°) of a base obtained from human semen and gives analyses of some salts derived from it. He considers it probable that this base is identical with Schreiner's base. Proof of this identity would only be afforded by the preparation of the characteristic phosphate, a task which apparently Wrede has not yet been able to accomplish. In the light of the work of H. W. Dudley, M. C. Rosenheim and O. Rosenheim [1924], however, it is evident that the base isolated by Wrede from semen is identical with that obtained by these authors as a phosphate from various animal tissues and which has been definitely identified with spermine. This conclusion is confirmed, in spite of various discrepancies in connection with melting points and analysis, by the agreement shown in the molecular weight of derivatives of the two bases.

A minute crystal of the chloroplatinate obtained from semen was also measured. Although its mass was only 0·0004 g., the crystal gave excellent reflections on the two-circle goniometer. The following measurements reveal a degree of approximation with the foregoing, which is but seldom exhibited by crystals of the same substance:

	m	*r*	*q*	*s*
ϕ	63° 7′	90° 0′	32° 45′	312° 14′
ρ	90 0	64 0	42 59	74 2

Summary.

1. Contrary to accepted statements in the literature, the "sperma crystals" were not discovered by Böttcher (1865), but had been previously described by Vauquelin (1791). The latter observer only "rediscovered," apparently independently, a fact originally observed by Leeuwenhoek in 1678.

2. Several new methods for the isolation of these crystals (= spermine phosphate) from semen are described, Schreiner's original method (1878) having been found unworkable.

3. Spermine occurs in testis, in which it is present as spermine phosphate.

4. Chemical and crystallographical evidence is brought which proves the identity of spermine from semen with the base obtained by similar methods from various animal organs and also from yeast.

My thanks are due to the Medical Research Council for permission to complete this work in the National Institute for Medical Research, to Dr Dale, the head of the Department concerned, and to Dr Dudley, who placed the facilities of his laboratory at my disposal. Mr W. W. Starling has given me much valuable assistance.

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(1) From Semen

(2) From Testis

(3) From Pancreas

(4) From Yeast

Fig. 2. Photomicrographs of Spermine Phosphate from various sources.

Magnification 50 ×

CLXVI. THE CHEMICAL CONSTITUTION OF SPERMINE. I. THE ISOLATION OF SPERMINE FROM ANIMAL TISSUES, AND THE PREPARATION OF ITS SALTS.

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An investigation of spermine has been proceeding for some time in these laboratories, and we propose to deal in this paper with the occurrence and isolation of this substance, and to describe some salts and derivatives which have enabled us to establish its chemical composition.

It has been pointed out in the preceding communication that, according to Schreiner [1878], the base is not specific to human semen, but is widely distributed in the animal organism. Schreiner's statement was discredited until one of us [M. C. Rosenheim, 1917] confirmed it and demonstrated the occurrence of spermine in the tissues of both male and female animals, and, further, succeeded in isolating it from yeast. It is clear, then, that we are dealing with a base of general biological interest, the presence of which has been overlooked for so long owing to the very small amounts occurring in the tissues. The yields of spermine phosphate which we have obtained from various tissues are as follows: testis (bull) 0·006 %, ovary (cow) 0·014 %, pancreas 0·025 %, brain 0·007 %, spleen 0·011 %, thymus 0·006 %, thyroid 0·003 %, and yeast 0·01 %. It is interesting to note that the highest yield was obtained from pancreas, and that a specimen of insulin "hydrochloride" prepared by the picric acid-acetone method yielded 7 %, the entire content in spermine of the pancreas from which the insulin was prepared[1]. We have failed to find it in defibrinated blood (14 l.), in serum and in cow's milk (16 l.).

Spermine is distinguished from all other naturally occurring bases hitherto described by the remarkable property of forming an extraordinarily insoluble phosphate. This salt is of the greatest historical importance. It is the most remarkable of all the salts of spermine, its characteristic crystalline form and great insolubility being the properties which led to its early detection (cf. the

[1] Mr H. P. Marks kindly tested the effect of spermine on the blood sugar of rabbits and found that it has no action.

preceding paper). In order to be certain that we were dealing with the same substance as that occurring in semen we considered it essential to isolate it in the form of this classical salt.

In spite of its great insolubility it is by no means easy to bring about the crystallisation of the phosphate unless special conditions are observed. These conditions were unfortunately not defined by Schreiner with the result that subsequent workers (Majert and Schmidt [1890] amongst others) failed to obtain the salt again and doubt has even been cast recently upon its existence. In fact, it seems unlikely that, with the exception of Poehl [1898], who for reasons of his own refrained from giving details, it had ever been prepared again until two of us (M. C. R. and O. R.), several years ago, determined the conditions necessary for its formation.

Ladenburg and Abel [1888] suggested that an essential constituent of the phosphate might be calcium, since they found this metal in a sample of the material which Schreiner sent them. They considered that the phosphate might possibly have the formula $(C_2H_6N)_4.Ca(PO_4)_2$, which agrees with Schreiner's analysis better than his own formula. We find, however, that the pure phosphate is free from calcium and that Schreiner's analysis of the phosphate is correct, although he misinterprets the results, assuming the formula $(C_2H_5N)_2.H_3PO_4.3H_2O$, which was supported by faulty analyses of the hydrochloride and chloroaurate.

There are several possible methods for isolating the material from tissues. That adopted in earlier work by two of us (M. C. R. and O. R.) consisted in the extraction of the tissue with hot acidulated water. From the filtrate, after treatment with lead acetate, a precipitate of phosphotungstates was obtained under the usual conditions. This precipitate was then extracted with acetone, which dissolves the bulk of the phosphotungstates, leaving a small insoluble fraction containing the whole of the spermine.

A considerable purification having been thus effected, the insoluble fraction was decomposed with baryta, and after removal of excess baryta from the filtrate it was neutralised with an appropriate quantity of phosphoric acid. The characteristic phosphate of spermine crystallised out, this process being hastened by the addition of alcohol.

Another method consisted in subjecting the tissue extract to Kossel's silver fractionation process, when spermine was found to pass into the lysine fraction, from which the phosphate was isolated as before.

The yield of spermine phosphate obtainable from tissues being so small, it was necessary, for the purpose of this research, to work up large quantities of material. In order to avoid the necessity of using such large amounts of phosphotungstic acid as would have been required in the processes just described, alternative methods of working up the concentrated extract were explored. A convenient process was found to consist in making this liquid alkaline and extracting continuously with butyl alcohol. Spermine passed into the alcohol and was precipitated by the addition of a solution of phospho-

tungstic acid in butyl alcohol. The precipitate was worked up by the same procedure as that employed in the case of the acetone-insoluble phosphotungstate described above. By these means a considerable saving of phosphotungstic acid was effected.

Spermine, which we have prepared in the free state, is a crystalline, odourless solid melting between 55° and 60°, and distilling *in vacuo* without decomposition. We find that it distils, somewhat slowly, with steam from strongly alkaline solution, and a very simple method of isolation has been based on this property. It is not only stable in hot concentrated alkali but also resists prolonged boiling with 30 % hydrochloric acid. The base, both as hydrochloride and in the free state, has been found to be optically inactive.

We have analysed the phosphate, hydrochloride, picrate, benzoyl derivative, chloroaurate and chloroplatinate. The entire series of analyses agree with the formula $C_5H_{14}N_2$, but the determination of the molecular weight of the benzoyl derivative by both Beckmann's and Rast's methods indicates that this formula should be approximately doubled. From these results, then, it becomes necessary to assign to spermine the molecular formula $C_{10}H_{26}N_4$.

Work on the constitution of spermine is being continued. The examination of the products of dry distillation of the hydrochloride, of exhaustive methylation of the base, of decomposition of the chloroaurate with magnesium has yielded valuable information in this connection which, along with other observations, will be reported in a later communication.

Experimental.

Preparation of spermine phosphate from pancreas.

(1) *Acetone method.* 5 kg. of minced fresh pancreas (ox) are poured into 10 litres of boiling water. The mixture is again heated to boiling and 30 cc. glacial acetic acid are added with vigorous stirring. After cooling the clear liquid is filtered off and as much fluid as possible is pressed out of the coagulated protein. Glacial acetic acid in the proportion of 5 cc. per litre of filtrate, and a saturated aqueous solution of lead acetate are added until no further precipitate is formed. The solution is then filtered and the excess of lead removed by cautious precipitation with sulphuric acid. After filtration the clear liquid is concentrated *in vacuo* (water bath at 45–50°) to about one-eighth of its original volume. Its content of sulphuric acid is brought up to 5 % and the bases are precipitated with phosphotungstic acid (25 % solution in 5 % sulphuric acid), about 500 g. being required for 5 kg. pancreas. The precipitate is filtered on a Buchner funnel, washed and pressed as dry as possible. It is then ground up in a mortar with acetone and transferred with more acetone to a flask, about 500 cc. acetone per kg. pancreas being used. A granular yellowish deposit settles rapidly in the dark brown solution. The precipitate is washed with acetone by decantation, filtered and dried. A yellowish powder, about 25 g.

from 1 kg. pancreas, is obtained. The phosphotungstate is decomposed in the usual way by grinding with saturated baryta water and solid baryta in a mortar. The filtrate is freed from baryta by CO_2 and subsequent warming to 70°.

The final filtrate, containing the base as carbonate, is accurately measured, one-third of it is withdrawn and titrated with 10 % phosphoric acid, using methyl orange as an indicator. The addition of the acid is continued carefully after the indicator has changed colour until the reaction is distinctly acid to sensitive Congo paper. The remaining two-thirds of the basic solution are then mixed with the acidified portion and the mixture will then be found to be amphoteric to litmus paper. Spermine phosphate crystallises out slowly in rosettes and single crystals on allowing the solution to stand for some hours.

In order to obtain the maximum yield, however, it is advisable to add one-third of the volume of alcohol when an immediate crystallisation of slender needles of spermine phosphate begins. The substance is recrystallised from 50–60 volumes of boiling water. Yield 0·9 g.

The procedure outlined in the last stage of the method has been evolved after many failures, which taught the necessity of adjusting the final reaction to neutrality, the slightest excess of acid or alkali preventing crystallisation. A colorimetric determination of the p_H of solutions of pure spermine phosphate demonstrated the fact that crystallisation only takes place within the narrow limits of p_H 6·8 to p_H 7·2, a fact which may be of physiological significance.

(2) *Butyl alcohol method.* An extract of 5 kg. pancreas is prepared in the same way as under (1), except that the lead treatment may be omitted. The concentrated extract is made alkaline by adding 25 cc. of 50 % NaOH solution per 100 cc. and the solution is extracted continuously with butyl alcohol after the method of Dakin for about 24 hours. To the butyl alcohol extract is added a 25 % solution of phosphotungstic acid in butyl alcohol until no further precipitate appears. The precipitate is allowed to settle and washed four times with acetone, either by decantation or by the centrifuge. After filtration and drying *in vacuo* the phosphotungstate is obtained as a white voluminous powder (about 10 g. from 1 kg. pancreas). Spermine phosphate is prepared from it in exactly the same manner as described under (1). Yield 1·0 g.

Of the alternative methods for the recovery of the base from its butyl alcoholic solution, which have been successfully applied, the following may be mentioned: (1) neutralisation with phosphoric acid under certain conditions, the phosphate being precipitated in an amorphous state, (2) precipitation of the picrate with a butyl alcoholic picric acid solution, and (3) extraction with dilute mineral acid, followed by precipitation with phosphotungstic acid.

(3) *Steam distillation.* 5 kg. of the tissue are treated with boiling water and acetic acid as in the first method. The filtered extract is evaporated *in vacuo* to as small a bulk as possible. Solid NaOH (50 g. per 100 cc. fluid) is added and the solution steam-distilled in the usual way. Distillation is continued until the distillate no longer gives a precipitate with Dragendorff's reagent, which indicates that all the spermine has been removed. The distillate

is then treated with phosphoric acid in the same manner as the baryta-free solution of the base in the preceding sections, and evaporated to about 100 cc. per kg. of pancreas taken. Alcohol is added to produce a concentration of 25 % when spermine phosphate crystallises out[1]. Yield 1·5 g.

The above methods have been applied with slight modifications to other tissues. Spermine phosphate has also been obtained from Lebedeff's and distiller's yeast, but not from baker's yeast. The commercial yeast extract "Marmite" contains about 0·04 %. From Liebig's Extract 0·01 % spermine phosphate was isolated. It was found that when the crystallisation of spermine phosphate is completed, the filtrates contain the soluble phosphate of another base which has not yet been identified. The amount is only one-tenth of that of spermine (about 2 g. from 100 kg. pancreas), its phosphotungstate is insoluble in acetone and it yields a well crystallised picrate (M.P. 210°). The soluble phosphate from yeast has been identified with putrescine (picrate, M.P. 264°; phenylisocyanate, M.P. 240°; benzoyl compound, M.P. 178°).

Properties and solubility of spermine phosphate.

On recrystallisation from water the substance is obtained in colourless crystals, insoluble in alcohol, ether and organic solvents, easily soluble in dilute acids and alkalies. The crystals slowly lose water of crystallisation over sulphuric acid, rapidly at 100°, becoming opaque. Their typical form is seen in the photomicrograph given in the preceding paper. When rapidly formed by the addition of alcohol, they appear as slender long needles. Occasionally they occur under these conditions as anisotropic spherocrystals, which quickly change into the usual form. The solubility of the pure salt was found to be 1 part in 100 parts of boiling water. At 20° its solubility in water is 0·037 %.

Behaviour of spermine phosphate in melting point determination.

The salt begins to soften at 227°, froths up the tube with evolution of gas and melts between 230–234°. At 240° the evolution of gas has stopped and a white deposit lines the tube. At 260–262° this deposit runs together with a slight evolution of gas. There is no discoloration during these changes.

Analysis. Water of crystallisation.

0·3110 g. four times recrystallised salt lost 0·0671 g. at 103°, whence $H_2O = 21{\cdot}57$ %.

Calculated for $C_{10}H_{26}N_4.2H_3PO_4.6H_2O$: 21·34 %.

0·2423 g. dry salt gave NH_3 (Kjeldahl) = 23·98 cc. $N/10$ H_2SO_4;

0·2239 g. „ „ 0·1250 g. $Mg_2P_2O_7$.

Found: N = 13·86 %, P = 15·56 %;

Calculated for $C_{10}H_{26}N_4.2H_3PO_4$: N = 14·07 %, P = 15·56 %.

[1] Ladenburg and Abel [1888] subjected a small sample of spermine, prepared by Schreiner himself, to steam distillation and assumed that the base is volatile. Since they were, however, unable to regenerate the phosphate from the distillate, their evidence is inconclusive.

Several preparations from testis and pancreas were analysed and gave identical results.

Spermine picrate.

2·78 g. spermine phosphate were dissolved in 13 cc. of cold 2*N* NaOH. The solution was filtered and diluted to 75 cc. with cold water. Saturated aqueous picric acid was then added until no further precipitate was formed. The picrate was filtered off and recrystallised from 1200 cc. boiling water. It separates in slender yellow needles often about 2 cm. in length. Yield 5·72 g.

It becomes black at about 242° and melts sharply with decomposition at 248–250°, contains no water of crystallisation and was analysed for picric acid by means of nitron.

0·0576 g. spermine picrate gave 0·1113 g. nitron picrate, whence picric acid = 81·78 %.

Calculated for $C_{10}H_{26}N_4.4C_6H_3O_7N_3$: 81·98 %.

Spermine hydrochloride.

5·7 g. spermine picrate were suspended in 60 cc. of a mixture of 75 cc. alcohol and 25 cc. 14 % aqueous HCl. The suspension was heated on a boiling water-bath for 10 minutes. The picrate did not go entirely into solution but was nevertheless decomposed and the liquid was now filled with a suspension of the white hydrochloride. This was poured into 500 cc. of dry acetone, the hydrochloride being thereby precipitated as a granular crystalline powder. This salt was filtered off and washed with acetone and finally with ether, until free from picric acid. The funnel containing the salt was transferred quickly to a vacuum desiccator and the adherent acetone and ether were removed by cautious evacuation in the presence of sulphuric acid. After about half-an-hour the spermine hydrochloride was obtained as a dry, white, non-hygroscopic powder. Yield 1·8 g.[1]

For recrystallisation it was dissolved in 12 cc. 10 % HCl and, after heating the solution on the water-bath, 40 cc. of boiling alcohol were added. The salt crystallised from this solution in short prismatic needles.

It is extremely soluble in water though not hygroscopic, very slightly soluble in hot methyl and ethyl alcohols and insoluble in acetone, ether and chloroform.

In the melting point tube it turns brown at 300–302°, and at 310° the mass liquefies and a slow evolution of gas is observed.

[1] This method of preparing hydrochlorides from picrates is quicker and less laborious than the usual process in which the picrate is decomposed with HCl in aqueous solution and the picric acid removed by shaking with ether. It also yields the hydrochloride in solid form instead of in solution with a large excess of HCl. With suitable modifications of the amounts of alcohol and aqueous HCl it can be applied in many cases to the preparation of hydrochlorides from picrates and probably from other salts such as picrolonates.

Analysis. The salt contains no water of crystallisation.

0·1028 g. gave 0·1675 g. AgCl: Cl = 40·60 %.

0·1146 g. „ 0·1451 g. CO_2; 0·0881 g. H_2O: C = 34·53 %; H = 8·54 %.

0·1159 g. „ NH_3 = 13·19 cc. *N*/10 H_2SO_4 (Kjeldahl): N = 15·93 %.

Calculated for $C_{10}H_{26}N_4.4HCl$:

C = 34·48 %; H = 8·62 %; N = 16·10 %; Cl = 40·80 %.

Spermine picrolonate.

0·010 g. spermine hydrochloride was dissolved in 1·5 cc. water, 1 cc. *N*/10 Na_2CO_3 solution was added and finally 2·5 cc. of a 2 % alcoholic solution of picrolonic acid. The picrolonate separated as a lemon-yellow crystalline powder: 0·036 g. was obtained. This was recrystallised from boiling water in which it is only sparingly soluble, 140 cc. being required. It crystallised in microscopic short prismatic needles of a dull, pale yellow colour. It melts with decomposition at 288–289°. Its solubility in water at 5° is approximately 1 part in 56,500. On account of its great insolubility this salt may prove useful for quantitative estimations.

Spermine chloroaurate.

To a 5 % solution of the hydrochloride in water, or of the phosphate in just sufficient hydrochloric acid to bring it into solution, 20 % gold chloride solution is added until no further precipitate appears. It can be recrystallised readily from 5 % aqueous hydrochloric acid, and is obtained in golden yellow, lustrous leaflets. It melts with decomposition at 225°.

Analysis. 0·1601 g. lost 0·0023 g. H_2O at 103°, and the dry salt gave 0·0796 g. Au on ignition, whence H_2O = 1·4 % (calculated for 1 mol. H_2O, 1·14 %), and Au = 50·44 %.

0·1699 g. dry salt gave 0·2500 g. AgCl: Cl = 36·40 %.

Calculated for $C_{10}H_{26}N_4.4HCl.4AuCl_3$: Au = 50·40 %; Cl = 36·38 %.

Spermine chloroplatinate.

0·25 g. of the phosphate is dissolved in 5 cc. water by the addition of 0·2 cc. 30 % HCl and 7 cc. 10 % chloroplatinic acid solution are added. Crystallisation takes place more slowly than in the case of the gold salt. The orange-yellow salt can be recrystallised easily from 5 % aqueous hydrochloric acid, and is obtained in large, well-formed crystals, which are crystallographically described in the preceding paper. The substance melts with decomposition at 242–245°.

Analysis. 0·1594 g. lost 0·0069 g. at 103°, and the dry salt gave 0·0577 g. Pt on ignition, whence H_2O = 4·33 %, and Pt = 37·91 %.

Calculated for $C_{10}H_{26}N_4.2H_2PtCl_6$: Pt = 38·20 %.

Benzoyl spermine.

1 g. spermine phosphate is dissolved in 40 cc. 8 % NaOH solution and shaken in a stoppered bottle with 6 cc. benzoyl chloride. A granular white deposit is rapidly formed. After half-an-hour 5 cc. of 8 % NaOH solution and 1 cc. benzoyl chloride are added and the mixture again shaken. The liquid is then poured off and the benzoyl compound is washed several times with water. It is then dissolved in 20 cc. warm alcohol and poured into 200 cc. water. A white sticky precipitate, which solidifies on standing, is deposited from the milky fluid. The water is decanted and the precipitate in the flask dried *in vacuo*. It is then boiled out several times with ligroin to remove benzoic acid and ethyl benzoate, dissolved in 70 cc. hot acetone, and ligroin added until a permanent turbidity is produced. It crystallises in woolly balls of fine needles. It may also be recrystallised from acetone or alcohol, diluted with an equal volume of water. Yield 0·98 g. The substance melts at 155°.

Analysis. 7·545 mg. gave 20·505 mg. CO_2 and 4·300 mg. H_2O[1],
4·232 mg. „ 0·36 cc. N at 17° and 716 mm.,
0·1508 g. „ NH_3 = 9·63 cc. $N/10$ H_2SO_4 (Kjeldahl):
C = 74·12 %; H = 6·38 %; N = 9·43 % and 8·94 %.

Calculated for $C_{10}H_{22}N_4(CO.C_6H_5)_4$:
C = 73·78 %; H = 6·79 %; N = 9·06 %.

Molecular weight determination.

(*a*) By Beckmann's method:

Solvent: 10·52 g. glacial acetic acid.

Weight of substance introduced:	(1) 0·0489 g.	(2) 0·0984 g.
Depression of freezing point:	0·030°.	0·060°.
Molecular weight:	604.	608.

(*b*) By Rast's method:

(1) 5·0 mg. in 50·6 mg. camphor. Depression: 6·5°. M.W. 608.
(2) 10·4 mg. in 100·8 mg. camphor. Depression: 6·5°. M.W. 634.

Calculated for $C_{10}H_{22}N_4.(CO.C_6H_5)_4$: M.W. = 618.

Phenylisocyanate derivative of spermine.

0·055 g. spermine hydrochloride is dissolved in 2 cc. water, to which is added 0·2 cc. 2*N* NaOH. After the addition of 0·2 cc. phenylisocyanate the liquid is stirred continuously and a sticky white precipitate is deposited. When the reaction of the liquid has become only faintly alkaline a further addition of 0·2 cc. 2*N* NaOH is made. After the reaction is over the sticky precipitate hardens and becomes brittle. It is rubbed up with a glass rod, filtered and washed with cold water. The solid is taken up in absolute alcohol, water is

[1] The micro-analyses were made ten years ago, when only small quantities of the product were available.

added until a permanent turbidity is produced; the liquid is then warmed and set aside to crystallise.

The phenylisocyanate derivative crystallises from alcohol in colourless clusters of needles. After four recrystallisations it melted at 179–180°.

Optical inactivity of spermine.

(*a*) As hydrochloride: 2 g. dissolved in 16 cc. water were examined in a 2 dm. tube and found to be optically inactive.

(*b*) As free base: 2·8 g. of spermine phosphate were dissolved in 15 cc. 2*N* NaOH, examined in a 2 dm. tube and found to be optically inactive.

Spermine itself is obtained by adding 50 % potash to a concentrated solution of the hydrochloride or to a suspension of the phosphate in water. The base is extracted with chloroform and remains, after the removal of the solvent *in vacuo*, as a colourless oil which rapidly solidifies to an aggregate of needle-shaped crystals, melting at 55–60°. On distillation *in vacuo* it boils without decomposition at about 150° under 5 mm. pressure. The base is possibly obtained under these conditions as a hydrate, a point which will be investigated later. It is easily soluble in water, ethyl and butyl alcohol, insoluble in ether, benzene and ligroin. On exposure to air it rapidly absorbs carbon dioxide and liquefies.

Beside the above-described derivatives we have also prepared the arsenate, which resembles closely the phosphate in crystalline form and solubility. The oxalate crystallises in leaflets, M.P. 225°. The sulphate, nitrate, acetate and carbonate crystallise well, but are very hygroscopic.

SUMMARY.

1. The base spermine has been isolated from the following animal organs: testis, ovary, pancreas, muscle, liver, brain, spleen, thymus and thyroid. It has also been obtained from yeast. The methods of isolation are described.

2. The free base is a solid which can be distilled *in vacuo* and is volatile with steam. It is optically inactive.

3. The conditions necessary for the preparation of its most characteristic salt, the phosphate, are laid down.

4. The phosphate, hydrochloride, picrate, chloroaurate, chloroplatinate and benzoyl derivative have been analysed. The results agree with an elementary composition of $C_5H_{14}N_2$. Other salts and derivatives are described.

5. Molecular weight determinations of the benzoyl derivative indicate a molecular weight approximately twice that given above. On this account the formula $C_{10}H_{26}N_4$ has been adopted.

This investigation was begun in 1910 by two of us (M. C. R. and O. R.) in the Physiological Laboratory, King's College, London, with the help of a

grant from the Government Grant Committee of the Royal Society. Resources then available only permitted work on a relatively small scale.

In the preparation of large quantities of spermine for the purposes of the research here presented, we have been greatly assisted by the British Drug Houses, Ltd., who have carried out large scale extractions for us. To them and to Mr F. H. Carr of their directorate our best thanks are due.

We are greatly indebted to Mr W. W. Starling for valuable and skilled assistance in connection with this work.

For references see preceding paper by O. Rosenheim, p 1253.

CLXVII. THE CARBOHYDRATE AND FAT METABOLISM OF YEAST. PART II. THE INFLUENCE OF PHOSPHATES ON THE STORAGE OF FAT AND CARBOHYDRATE IN THE CELL.

By IDA SMEDLEY MACLEAN and DOROTHY HOFFERT.

(*Received October 6th, 1924.*)

It has been clearly established that fat is formed by the yeast cell from carbohydrate; in a previous communication [1923] we brought forward evidence that the addition of alkali phosphates to a solution of carbohydrate in which yeast was incubated produced an increase in the amount of fat stored up by the yeast cell. We also found that if the proportion of sugar was varied in a medium containing a certain definite concentration of phosphate, the amount of phosphate taken up by the yeast cell varied with the concentration of the sugar in the medium.

Many years ago Harden and Young [1906] showed that the initial rate of fermentation with yeast juice was much increased by the addition of phosphates to the fermenting liquid. In the work described in our previous communication, the experiments were all carried out under carefully controlled conditions in which the incubation of the yeast in the solution under investigation was allowed to proceed for 46 hours, and the composition of the yeast then determined. It seemed possible that the phosphate might also act here, as it does in the yeast juice fermentation, by increasing the rate of change in the transformation of the carbohydrate. If this were the case the total amount of fat in a yeast incubated in a solution of sugar without phosphate would, if the experiment were continued, approach to that stored in a yeast incubated in a sugar solution to which phosphates had been added.

Incubation in glucose solution.

When yeast is incubated in a 4 % solution of a fermentable sugar, the sugar is continually removed from the medium, and at the end of three days very little sugar remains in the solution; in this case the rate of fat formation steadily decreases, most of the fat being made in the first 24 hours. If the original yeast contains a high percentage of carbohydrate a preliminary fall in carbohydrate content may take place followed by a rise; if the original carbohydrate content is low the maximum amount of carbohydrate seems to be reached in from 24 to 36 hours (see Table I).

Both in the presence and absence of phosphates in the fermenting liquid the rate of fat formation was found to be highest at the beginning of the experiment.

Table I. *Yeast incubated in a 4 % solution of glucose.*

(G. per 12·5 g. of original yeast.)

	(*a*) Without phosphate			(*b*) With phosphate			
Hours	Prot.	Carb.	Fat	Prot.	Carb.	Fat.	Mg. phosphorus
Original	1·30	1·03	0·075	1·34	0·82	0·091	65·4
12	1·25	1·24	0·342	1·30	1·08	0·496	78·2
24	1·22	1·25	0·514	1·15	1·36	0·665	82·0
36	1·24	1·09	0·602	1·19	1·26	1·047	101·1
Original	1·30	1·00	0·056	1·24	1·43	0·052	66·5
24	—	1·18	0·436	1·19	1·14	0·656	107·5
48	1·19	1·14	0·454	1·15	1·21	0·669	122·9
72	1·20	1·07	0·542	1·17	1·16	0·911	125·2

We then studied the rate at which fat was stored up by the yeast cell when incubated in a solution of sugar, the sugar of which was periodically renewed, so that an excess of this substance was always present. Experiments were carried out both with and without the addition of phosphates.

The method employed was to incubate for 46 hours several quantities of 12·5 g. of yeast each in a litre of 4 % glucose solution, oxygenated throughout the experiment. At the end of this time usually less than 1 % of sugar remained. The samples of yeast were then filtered off and each again incubated in a litre of sugar solution for 46 hours just as before, one sample however being retained for analysis. At the end of a further period of 46 hours the samples were again filtered off, one retained for analysis, and the others returned as before to fresh quantities of sugar solution; the process was again repeated. In this way a comparison was made of the composition of the samples after incubation for 2, 4, 6, 8 and 10 days respectively.

A similar set of experiments was carried out, in which the medium contained in addition to the 4 % of glucose a mixture of alkali phosphates (0·3692 g. di-sodium phosphate and 0·0286 g. potassium di-hydrogen phosphate %), the mixture of sugar and phosphates being renewed every 46 hours.

The analyses obtained in these experiments are shown in Table II.

Samples of yeast obtained from the brewery on different days showed considerable variations in their fat-forming power, which did not appear to be connected with the age of the yeast; we also observed that when a sample of yeast had been kept overnight in the cold room it became less active in storing fat.

The yeast used in Exp. K was one of the most efficient we tested in its power of forming fat. The fat extracted from this yeast after it had been incubated in the glucose solution smelt strongly of crotonaldehyde. The residues were left in the Soxhlet thimbles for some days exposed to air and light, and were then again extracted with ether. After evaporating off the ether on a

Table II. *Yeast incubated in glucose solution* (4 %), *renewed every* 48 *hours.*

(G. per 12·5 g. of original yeast.)

No. of days incubated	(*a*) With phosphate Prot.	Carb.	Fat	No. of days incubated	(*b*) Without phosphate Prot.	Carb.	Fat	P. mg.
Exp. A				*Exp.* F				
Original	1·42	1·51	0·056	Original	1·41	0·88	0·109	50
2	1·34	1·36	0·292	2	1·24	1·02	0·616	92
4	1·28	1·39	0·690	4	1·20	2·02	1·080	121
6	1·30	1·67	1·080	6	1·18	2·39	1·146	135
Exp. B				*Exp.* G				
Original	1·41	1·48	0·095	Original	1·36	1·28	0·061	75
2	—	—	—	2	1·36	1·26	0·490	122
4	1·40	1·57	0·722	4	1·28	1·79	0·853	156
6	—	—	—	6	1·32	2·50	1·112	177
8	1·35	1·87	1·321	8	—	—	—	—
Exp. C				*Exp.* H				
Original	1·37	1·16	0·110	Original	1·37	1·16	0·110	—
6	1·39	1·89	1·528	6	1·20	2·93	1·730	—
8	1·19	2·08	1·661					
10	1·23	2·25	1·568					
Exp. D				*Exp* K				
				Original	1·40	0·97	0·090	58
				4	1·36	2·12	1·308	109
				6	1·37	2·43	1·708	135
				8	1·37	2·87	2·279	137
Exp. E				*Exp.* L				
Original	1·21	1·00	0·088	Original	1·21	1·00	0·088	68
2	1·12	1·10	0·321	2	1·18	1·33	0·433	109
4	1·18	1·30	0·549	4	1·21	1·53	1·129	135
6	1·26	1·42	0·823	6	1·25	1·72	1·279	137

In Exps. C and H and in E and L the same specimen of yeast was added so that the results in each pair of these experiments are strictly comparable.

water-bath a small quantity of an unsaturated aldehyde, in odour and volatility strongly resembling crotonaldehyde, was left. During a long series of some hundreds of experiments which we have carried out, we have twice noted the presence of an unsaturated lower fatty aldehyde in the fat, one in the experiment above referred to, in which a mixture of glucose with phosphate was fermented, and the other in which a fructose solution had been fermented. So far we have not been able to determine the exact conditions to which the presence of the aldehyde may be ascribed, but we are inclined to think it is associated with some slight variation in the yeast. In both cases, the yeast produced rather specially large amounts of fat. The result seems to us of considerable importance, and suggests the occurrence of an unsaturated aldehyde as an intermediate stage in the chain of reactions by which carbohydrate is converted to fat, which would be expected to occur if the fat is built up by the condensation of aldehyde with pyruvic acid [Smedley and Lubrzynska, 1913], or with other aldehydes [Magnus-Lévy, 1902].

The results obtained show that the rate of fat formation is increased by

adding phosphates to the glucose-containing medium in which the yeast is incubated. In the absence of phosphates, when the experiment is continued for several days with the addition of fresh quantities of glucose to the medium at the end of every 46 hours, the rate of increase seems to be more or less constant until a limit is reached when further incubation produces no increase of fat. If, however, the fermenting liquid contains phosphate, the rate of increase is higher at the beginning of the experiment and then diminishes.

In those experiments where the same yeast is used both with and without phosphates, the total amount of fat is always greater in the yeast which has been incubated in the phosphate-containing medium. On the whole, therefore, the evidence shows that not only is the rate of fat-formation stimulated by the addition of phosphates to the medium, but the total amount of fat deposited in the yeast is greater than that stored in the same specimen of yeast incubated in a sugar solution of similar strength, but containing no phosphate.

Incubation in fructose solution.

The influence of phosphates in increasing the total amount of fat formed is shown most strikingly when equal amounts of the same sample of yeast are incubated in a 4 % oxygenated fructose solution respectively with and without the addition of phosphates.

Table III. *Showing composition of yeast after incubation in* 4 % *fructose solution.*

(G. per 12·5 g. of original yeast)

	Days	Prot.	Carb.	Fat		Prot.	Carb.	Fat	P. mg.
Exp. A	—	1·14	1·36	0·068	*Exp.* C	1·14	1·36	0·068	71
	2	1·09	1·15	0·273		1·11	1·43	0·587	123
	4	1·14	1·35	0·541		1·04	1·92	1·069	130
	6	1·14	1·58	0·717		1·13	2·58	1·470	143
	8	1·15	1·70	0·743		1·04	2·23	1·360	140
	10	1·08	1·78	0·988		1·12	2·62	1·974	145
Exp. B	—	1·40	0·47	0·101	*Exp.* D	1·40	0·47	0·101	71
	2	1·26	1·32	0·390		1·40	1·39	0·703	116
	4	1·25	1·39	0·608		1·21	2·03	1·119	134
	6	—	1·72	0·680		1·17	2·18	1·335	146

The experiment was arranged so that the medium could be renewed every 48 hours, and samples of the yeast analysed after incubation for periods of 2, 4, 6, 8 and 10 days respectively in the solutions of fructose, with and without the addition of phosphates. In each case the effect of the addition of the phosphate to the fructose solution was to double, almost exactly, the amount of fat in the yeast when compared with the control without the addition of phosphates. Although the amount of fat was greater in the yeast incubated in the glucose-phosphate solution than in the yeast incubated in the glucose solution, there was no such regular relation between these amounts as we observed when the yeasts were incubated in the corresponding fructose solutions.

The results obtained from these experiments with fructose strongly support the view that phosphate and fructose are both concerned in forming some intermediate substance which constitutes an essential stage in the conversion of sugar to fat; for the further transformation of this intermediate substance to fat, the presence of a supply of oxygen appears to be essential, and there is some evidence which suggests that an unsaturated aldehyde is formed at a later stage in the transformation.

Storage of carbohydrate.

Both with the yeast incubated in fructose and glucose solutions the addition of phosphate to the medium exercises a pronounced effect on the amount of carbohydrate stored by the cell. With each successive period of incubation in the phosphate and hexose medium, the amount of carbohydrate in the yeast increases, the amount being always greater than that contained in the yeast incubated in the hexose solution without the addition of phosphate. When the experiments are carried out in the manner now described, *i.e.* continued for periods of from 6 to 10 days with frequent renewals of the sugar in the medium, though fructose is more efficient in producing storage of fat than is a corresponding solution of glucose, no constant difference in their influence on the storage of carbohydrate could be detected.

The action of yeast on solutions of hexosephosphate.

When a solution containing sugar and sodium phosphate is fermented by yeast juice large quantities of hexosephosphate can be obtained from the fermentation liquid, but if the fermentation be carried out by living yeast the medium does not contain hexosephosphate.

Neuberg, Levite and Schwenk [1917] regard the formation of hexosephosphate as a pathological phenomenon, but the evidence which we brought forward [1923] that the absorption of phosphate by the yeast cell depends on the concentration of sugar in the medium, supports the view of Harden and Young [1905] that the formation of hexosephosphate is a normal stage in the fermentation process. From the fact that hexosephosphate is found in the fermentation liquid only when the fermentation is carried out with yeast juice, and not when it is carried out with the living yeast cell, it would appear that while the molecules of hexose and sodium phosphate can pass through the wall of the cell, the larger molecules of hexosephosphate are unable to do so. The yeast contains an enzyme capable of synthesising hexosephosphate but incapable of passing through the wall of the cell, and it is only when the cell is broken up and the enzyme obtained in the yeast juice that the hexosephosphate is found in the fermentation liquid.

In order further to test this conclusion we compared the action of yeast on solutions of sodium hexosephosphate, with its action on solutions of the corresponding amounts of glucose and sodium phosphate. In a 4·8 % solution

the action of hexosediphosphate was found to be deleterious to the yeast cell, a loss of carbohydrate and an increase of fat were noted similar to those recorded when yeast is incubated in water, but the losses of protein and of phosphorus were considerably greater than when yeast is incubated in water alone. When a more dilute solution of hexosephosphate was used there was little loss of protein, but there was no increase of phosphorus such as that obtained when yeast is incubated in a solution of similar concentration containing glucose and sodium phosphate.

Table IV. *Composition of yeast after incubation in a solution of hexosephosphate and in solutions of glucose with alkali phosphates.*

	Protein	Fat	Carbohydrate	P. mg.
Original yeast	1·28	0·097	1·01	63·5
Na hexosediphosphate (4·8 %)	0·90	0·164	0·28	32·1
Corresponding glucose (2 %) and phosphate	1·30	0·416	1·05	81·3
Original yeast	1·34	0·085	0·83	65·9
Na hexosediphosphate (1·8 %)	1·32	0·218	0·16	62·5
Corresponding glucose (0·8 %) and phosphate	1·28	0·356	0·55	75·5
Original yeast	1·25	0·098	0·40	73·7
Na hexosediphosphate (1·16%)	1·07	0·175	0·33	73·5
Corresponding glucose (0·48 %) and phosphate	1·24	0·268	0·49	74·1
Original yeast	1·36	0·079	0·40	61·0
Na hexosemonophosphate (1·2 %)	1·24	0·137	0·15	55·0
Corresponding glucose (0·75 %) and phosphate	1·34	0·482	0·60	77·0
Phosphate alone (0·37 % NaH_2PO_4) (0·03 % K_2HPO_4)	1·06	0·129	0·19	—

The increase of fat observed after the yeast had been incubated in the dilute hexosephosphate solution, was much less than after incubation in a solution of a mixture of glucose and phosphate of similar concentration. The amount of fat after incubation in the dilute hexosephosphate solution was of the same order as that obtained after incubation in a solution of sodium phosphate alone, and probably came therefore from the carbohydrate originally stored in the cell but which disappears during the period of incubation.

These experiments support the view that the hexosephosphate molecules are not able to pass through the wall of the yeast cell but that glucose and phosphate molecules pass separately into the cell and are there combined.

We are indebted to the Food Investigation Board of the Department of Scientific and Industrial Research for giving us the opportunity of carrying out this work.

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CLXVIII. FURTHER OBSERVATIONS ON THE INFLUENCE OF SUNLIGHT UPON THE GROWTH-PROMOTING AND ANTI-RACHITIC PROPERTIES OF COW'S MILK.

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(*Received October 17th, 1924.*)

In a recent communication [Luce 1924], it was shown that during summer, when the cow was pasture-fed, the anti-rachitic value of the cow's milk was increased above that of the milk afforded by the same animal when stall-fed and kept in the dark. The growth-promoting value of the milk was also shown to vary within wide limits which corresponded to variations in the animal's diet. Upon a diet of fresh green grass she yielded a milk superior in growth-promoting quality to that obtained when her diet was a dry fodder, deficient in fat-soluble vitamins. An attempt was also made to determine the effect on the anti-rachitic and growth-promoting value of the milk of exposing the cow to varying degrees of illumination, whilst keeping her upon a constant diet, but the results obtained up to the time of publication were not conclusive.

Since then the experiments have been continued by keeping the animal for eight months on the diet deficient in fat-soluble vitamins and comparing the value of the milk given respectively when in a stall under winter conditions and when exposed to sunlight of summer intensity.

The cow was of Jersey breed and was kept under carefully controlled conditions at the Serum Farm of the Lister Institute at Elstree, Herts., for eighteen months previous to, and during, the present observations. My acknowledgements are due to Dr MacConkey and his staff, who, during the whole period, kindly undertook the care, management and feeding of the cow as well as the collection and pasteurisation of the various milk samples.

Method of Experiment.

The present experiment was in effect a continuation of the last period (Period V), described in the original paper [1924, pp. 718–23]. The same cow was used, and the same technique employed for evaluating the samples of milk. Three samples were tested between October 23rd, 1923, and July 2nd, 1924. During this time the diet of the cow was unaltered, and was deficient in fat-soluble vitamins. It consisted of a daily ration of white maize meal $3\frac{3}{4}$ lbs.,

gluten meal 3 lbs., oats and barley (crushed) 3 lbs., mangolds 50 lbs., barley straw *ad lib.* The following samples of milk were tested:

1. Sample XV, representing the pooled milk afforded from December 12th to 19th, 1923, after the cow had received the above diet for two months and been kept out of doors in a ploughed enclosure by day and in her stall by night. The results obtained with this milk were given in the first communication and are resumed here in Table I and Fig. 1 (*a*) for comparison.

2. Sample XVI, pooled milk afforded from April 9th to 16th, 1924. Owing to the weather it was found necessary to keep the cow in stall from December 20th, 1923, to April 16th, 1924. The stall, however, was kept as light as possible by removing the upper half of the door by day.

3. Sample XVII, pooled milk afforded from June 25th to July 2nd, 1924. On April 17th, 1924, the cow was again let out into the ploughed enclosure where she remained exposed to all the available sunlight till July 2nd, 1924.

This last interval included several periods of very fine weather: April 17th to 21st (average sunshine 10 hours daily), and June 14th to end of the period, July 2nd (average sunshine 9 hours daily). During the whole division (77 days) the average number of hours of sunshine daily was 6·3. The incidence of sunlight upon the cow was therefore in marked contrast to that of the periods preceding the taking of samples XV and XVI, where the average number of hours' sunshine daily was about three, and, moreover, the animal was for the most part kept indoors.

Variations in the intensity of sunlight received by the cow during the three divisions of Period V.

The intensity of sunlight during the three divisions of Period V, at the end of which samples XV, XVI and XVII respectively were tested, can be roughly gauged by a study of the Meteorological Records taken at Rothamsted Experimental Station, Herts. These records show that during the first division, October 23rd, 1923, to December 19th, 1923 (sample XV), the light was of a gradually decreasing intensity: 5·6 hours per day are recorded during the last week in October, 1923, an average of 3·4 hours per day during November and 1·3 hours during December. During the second division, it increased from an average of 1·9 hours per day during January and February, 1924, to an average of 5 hours per day during March and April, but during this time the cow, as already stated, was not out in the open. During the third division the incidence of sunshine was 6·1 hours daily in May and 6·6 hours per day in June. During this time the cow was kept out of doors.

The following table gives the total number of hours of sunshine recorded in each of the three divisions of Period V.

Table I.

No.	Period V	Cow	Sample tested at end of period	No. of days	Total hours of sunshine	Average sunshine per day (hours)
1	Oct. 23–Dec. 19, 1923	In ploughed enclosure	XV	58	167	2·9
2	Dec. 20–April 16, 1924	In stall	XVI	118	374	3·2
3	April 17–July 2, 1924	In ploughed enclosure	XVII	76	482	6·3

The growth-promoting value of the milk.

The criterion used for estimating the growth-promoting value of the milk was the same as that adopted previously, viz. the reciprocal of the daily dose necessary to induce young rats, which had ceased to grow on the basal "– A" diet, to resume growth in body weight to the extent of 10–12 g. per week, the period of observation being 35 days. With the three samples of milk tested (XV, XVI, XVII) this dose was found to be between 5 and 8 cc. daily. The milk therefore contained 20–12 growth units per 100 cc. and no difference in value could be detected between the three samples, see Protocols F—H.

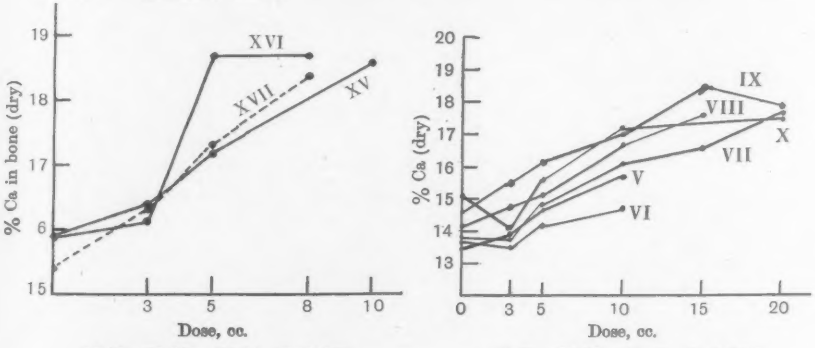
It is evident therefore that the amount of sunlight received by the cow during the latter part of the time was ineffective in raising the growth value of the milk. This value, however, did not fall to the lowest level (10–6 units per 100 cc.), obtained during Period II (October, 1922–July, 1923) when the cow was fed on the same diet but kept in a dark stall.

These results confirm those obtained by Hume [1923] who failed to raise the growth-promoting value of the milk by exposing a cow, fed on cereals, roots and straw, to summer sunshine.

Anti-rachitic properties of the milk.

(*a*) *Evidence from the determination of calcium-content in the bones of rats used in the titration of samples XV, XVI, XVII. Period V.*

The results obtained by estimating the percentage of calcium (on dry weight) in bones of rats (femur, tibia, and fibula), are displayed in Fig. 1 (*a*) (and Protocols F–H). The increase in calcium content, as compared with



(*a*) Dry Fodder; Light. Period V. (*b*) Dry Fodder; Dark. Period II.

Fig. 1. Average calcium content (percentage reckoned on dry weight of bone) in bones of rats receiving various daily supplementary doses of milk, compared with that in control animals on basal (– A) diet alone.

(*a*) Milk samples XV–XVII, Period V: cow on dry fodder and exposed to light.

(*b*) Milk samples V–X, Period II: cow on dry fodder but in a darkened stall (Fig. taken from Luce, 1924, Fig. 2, p. 727).

Protocol F. Period V. Sample XV. Dry Fodder; Light.

Rat	Sex	Family	Dose	Initial wt	Final wt	Post-mortem evidence of rickets	In bones % Ca (dry)	Histological diagnosis
840	♂	1	No milk	92	106	–	15·6	Osteoporosis + slt rickets
863	♂♀	2	„	79	108	–	15·3	„ „
826	♀	3	„	55	—	–	Died & eaten	Osteoporosis
855	♀	4	„	72	87	–	16·9	„
						Av. no milk	15·9	
825	♀	3	3 cc.	50	77	–	14·9	Osteoporosis
828	♀♀	3	3 „	79	88	–	17·2	„
866	♂	2	3 „	78	100	–	17·2	„
						Av. 3 cc.	16·4	
832	♂	3	5 cc.	69	103	–	15·6	Slight osteoporosis
845	♀	1	5 „	73	124	–	18·4	Normal
861	♂♂	2	5 „	65	105	–	17·0	Slight osteoporosis
867	♀	4	5 „	76	117	–	17·8	„
						Av. 5 cc.	17·2	
853	♀	4	10 cc.	63	107	–	18·5	Normal
851	♀♀	4	10 „	69	110	–	20·5	„
868	♂	2	10 „	62	126	–	16·9	„
						Av. 10 cc.	18·6	

Protocol G. Period V. Sample XVI. Dry Fodder; Light –.

Rat	Sex	Family	Dose	Initial wt	Final wt	Post mortem evidence of rickets	In bones % Ca (dry)	Histological diagnosis
957	♀	1	No milk	67	64	–	15·1	Osteoporosis
960	♂	1	„	72	53	–	17·6	„
963	♂♀	2	„	55	49	+	13·5	Slt rickets + osteoporosis
970	♂♀	2	„	56	52	+	15·9	„ „
935	♂♀	3	„	50	82	+	16·7	„ „
934	♂	3	„	68	120	–	17·0	Osteoporosis
						Av. no milk	15·9	
951	♀	1	3 cc.	73	89	–	16·5	Osteoporosis
936	♀♀	3	3 „	60	117	–	17·8	Slight osteoporosis
953	♂♂	1	3 „	69	105	–	16·3	„
962	♂	2	3 „	60	80	–	13·9	Infraction osteoporosis
						Av. 3 cc.	16·1	
939	♀	3	5 cc.	54	110	–	18·4	Nearly normal
952	♀♀	1	5 „	74	115	–	18·6	Slight osteoporosis
955	♂	1	5 „	75	116	–	18·9	„
966	♂	2	5 „	67	98	–	18·9	„
						Av. 5 cc.	18·7	
933	♀	3	8 cc.	73	136	–	18·9	Slight osteoporosis
938	♀♀	3	8 „	70	131	–	20·6	Nearly normal
959	♂	1	8 „	75	119	–	18·4	Slight osteoporosis
964	♂	2	8 „	58	106	–	16·9	„
						Av. 8 cc.	18·7	

Protocol H. Period V. Sample XVII. Dry Fodder; Light +.

Rat	Sex	Family	Dose	Initial wt	Final wt	Post-mortem evidence of rickets	In bones % Ca (dry)	Histological diagnosis
974	♀	1	No milk	73	76	–	15·2	Osteoporosis
975	♂♀	1	„	73	52	Died	16·9	„
978	♀	2	„	72	57	„	14·9	„
981	♂♀	2	„	82	57	–	15·8	„
988	♀♀	3	„	66	53	–	13·7	„
989	♂	3	„	61	55	Died	15·9	„
						Av. control	15·4	
973	♀	1	3 cc.	64	83	–	15·3	Osteoporosis
980	♀	2	3 „	72	88	–	16·7	„
983	♂♀	2	3 „	67	—	Died	—	—
987	♀	3	3 „	48	79	–	17·0	Slight osteoporosis
						Av. 3 cc.	16·3	
971	♂	1	5 cc.	70	119	–	15·8	Osteoporosis
982	♂♀	2	5 „	73	103	–	17·7	Slight osteoporosis
984	♀	2	5 „	53	87	–	17·6	„
990	♀	3	5 „	63	117	–	18·0	„
						Av. 5 cc.	17·3	
970	♂	1	8 cc.	82	128	–	16·6	Nearly normal
977	♀♀	2	8 „	74	110	–	19·8	„
985	♀	3	8 „	62	105	–	18·9	„
						Av. 8 cc.	18·4	

control animals, in bones of rats receiving 3–5 cc. doses of milk daily of samples XV–XVII is seen from Fig. 1 (*a*) to be slightly greater than that obtained with rats receiving the same supplements of milk from Period II (samples V, VI, VII, VIII, IX and X, Fig. 1 (*b*)) when the cow was fed on the same diet, but kept in a specially darkened stall [1924, p. 727].

In order to interpret correctly the results obtained, it is necessary to consider not only the effect of sunlight on the cow, but the effect of varying conditions of light in the laboratory on the rats used for titrating the milk, and of any possible variation there may have been in the reserves of the anti-rachitic factor possessed by these animals.

It was sometimes noticed that control animals, receiving no milk, appeared to grow better and to show a higher calcium content of bone in summer when the illumination of the rooms was at a maximum [1924, p. 726] than in winter. In both seasons the light passed through window glass. Allowance has been made for this possible source of error. In Fig. 2 is shown the *average percentage increase in calcification above that of the controls*, in animals receiving 3, 5 and 10 cc. doses daily of the milk of Period II (samples V, VI, VII, VIII, IX and X) compared with that of Period V (samples XV, XVI and XVII). Each point in the lowest curve (Period II) of this figure represents the average percentage increase over controls in the case of 24 rats, and summarises the results of tests of six samples with four rats on each dose: 3 cc., 5 cc., 10 cc. respectively. In the middle curve (Period V) each average corresponds to

results obtained with twelve rats, as three samples of milk only were tested in this period.

From Figs. 1 and 2 it would appear that the milk from a cow exposed to light possesses *a slightly but definitely higher calcium-depositing power* than that from the same animal on the same diet, but shut up in a dark stall.

In Fig. 2 a curve showing results obtained during Period IV when the

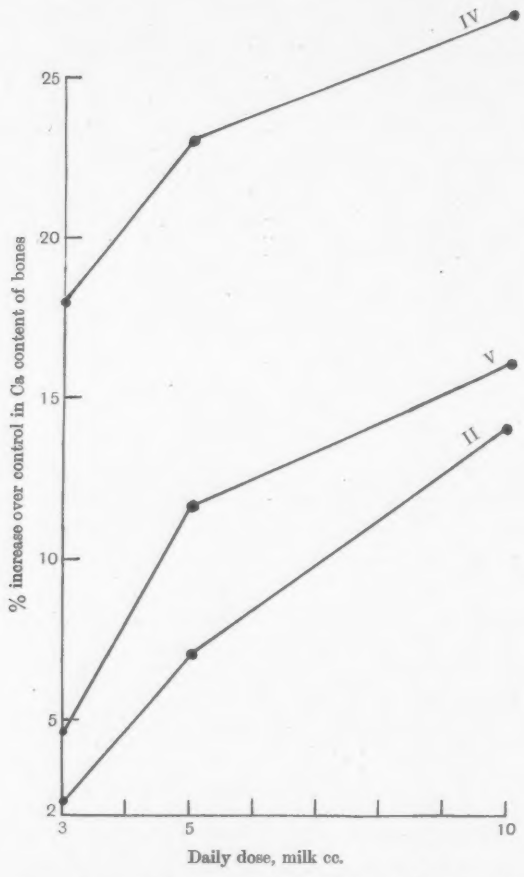


Fig. 2. Mean percentage increase over control animals in bone-calcification of rats receiving:

Curve II. 3, 5 and 10 cc. daily of milk taken in Period II. Dry Fodder; Dark (milk samples V, VI, VII, VIII, IX, X).

Curve V. 3, 5 and 10 cc. daily of milk taken in Period V. Dry Fodder; Light (milk samples XV, XVI, XVII).

Curve IV. 3, 5 and 10 cc. daily of milk ken during Period IV. Pasture-feeding (milk samples XIII, XIV).

cow was pasture-fed, is included for purposes of comparison. Its significance is discussed later.

(b) Evidence from post mortem and histological observations of rats used in titration of samples XV, XVI, XVII.

In the group of rats used for the titration of sample XV, rickets occurred in two out of four control animals, but in none of the three animals receiving 3 cc. of milk; the rats receiving higher doses showed by histological examination of the costo-chondral junction either a normal picture or one of slight osteoporosis (Protocol F).

With the rats used in the titration of sample XVI the results obtained were similar. A histological picture of rickets was obtained in three out of six control animals, but the milk-fed animals receiving doses of 3 cc. and upwards showed no evidence of rickets (Protocol G).

The six control animals used in the titration of sample XVII gave a histological picture of osteoporosis only, and none of the rats receiving 3 cc. of milk and upwards showed evidence of rickets (Protocol H).

Table II. *Incidence of rickets in the rats used in titrating milk samples taken during Period II (Dry Fodder; Dark) and Period V (Dry Fodder; Light) respectively.*

	Controls		Milk 3 cc.		Milk 5 cc.	
Period	Total	No. showing rickets	Total	No. showing rickets	Total	No. showing rickets
II	18	11	16	15	15	14
V	16	5	11	0	12	0

Table II shows that the incidence of rickets in the control animals receiving no milk is twice as great in Period II as in Period V. The rats receiving 3 and 5 cc. of the milk of Period II showed *a higher percentage incidence of rickets than the controls.*

In the rats used for testing milk of Period V, rickets occurred in none of the animals receiving 3 cc. milk, so that the difference between the two periods in the incidence of histological rickets in the milk-fed animals is very marked, *being much greater during Period II when the cow was kept in the dark.*

(c) Evidence from experiments with rats using McCollum's rickets-producing diet No. 3143.

Three families of rats, 22 in all, were used for testing the rickets-preventing value of sample XVII, taken at the *end* of Period V. As is described in the original paper, rats were fed on McCollum's No. 3143 rickets-producing diet for a period of 28 days with milk supplements of 3, 5, 8 and 10 cc. daily, whilst two rats from each litter received no milk and acted as controls. Results of this experiment are given in Table III. The protective dose with this milk was found to be 10 cc., the same result as that obtained with sample XV at the *beginning* of Period V (see previous paper, Table V, p. 730).

Table III. *Anti-rachitic value of milk at end of Period V. Sample XVII (Dry Fodder; Light). Rats on McCollum's 3143 diet for 28 days with milk supplements.*

Rat	Sex	Family	Dose	Initial weight	Final weight	Post-mortem evidence of rickets	In bones % Ca (dry)	Histological diagnosis
999	♂	1	No milk	48	81	+ + +	9·0	Severe rickets
1000	♀	1	„	42	62	+ + +	9·9	„
1007	♂	2	„	50	93	+ +	12·1	„
1008	♀	2	„	47	73	+ + +	11·6	„
1019	♂	3	„	52	85	+ +	12·5	Pronounced rickets
1020	♀	3	„	47	80	+ + +	10·6	Severe rickets
						Av. control	10·9	
1001	♂	1	3 cc.	45	100	+ + +	11·9	Severe rickets
1009	♀	2	3 „	46	92	+	15·9	Mod. rickets
1010	♀	2	3 „	51	108	+	15·1	„
1021	♂	3	3 „	47	87	+ +	13·4	Pronounced rickets
						Av. 3 cc.	14·0	
1002	♀	1	5 cc.	48	109	+ +	13·2	Pronounced rickets
1003	♂	1	5 „	46	90	+	15·0	„
1011	♂	2	5 „	15	116	+	14·5	„
1022	♀	3	5 „	46	93	+ +	13·5	„
						Av. 5 cc.	14·1	
1023	♂	3	8 cc.	44	113	? +	16·0	Slight rickets
1004	♀	1	8 „	46	111	? +	17·0	„
1006	♂	1	8 „	44	89	? −	17·7	„
1013	♀	2	8 „	47	126	? −	17·3	„
						Av. 8 cc.	17·0	
1012	♀	2	10 cc.*	45	108	−	18·6	Nearly normal
1005	♂	1	10 „	45	93	−	18·0	„
1024	♂	3	10 „	45	125	−	18·5	„
1014	♀	2	10 „	50	131	−	18·5	„
						Av. 10 cc.	18·4	

* Protective dose.

It is evident from this that exposure of the cow to direct sunlight for an average of 6·4 hours daily for 77 days failed to raise the anti-rachitic value of milk to a degree that could be detected by this severe test.

At the same time the anti-rachitic value of this milk did not fall to the level of that tested at the end of Period II (Dry Fodder; Dark) and Period III (Green Fodder; Dark) when 15–20 cc. was found to be the dose necessary for protection against rickets.

Discussion of Results.

The *growth-promoting value of milk* appears to depend principally, if not entirely, on the diet of the cow. After feeding her for a period of three months on a diet deficient in fat-soluble vitamins, the growth value of her milk fell to a low level and remained at this level for five months longer, in spite of the

fact that during part of this time she was exposed to sunshine of summer intensity.

A slightly lower level of growth-promoting value was reached in a previous experiment, when, on the same diet, the animal was shut up in a dark stall. It is possible that this difference may be within the limits of experimental error, and the conclusion reached, in confirmation of the previous work and of the results of other investigators, is that *variations in the growth-promoting power of the milk depend upon the presence or absence of fat-soluble vitamins in the diet of the cow.*

The anti-rachitic value of the milk. There is some evidence from these experiments that *exposure of the cow to sunlight plays a part in raising the anti-rachitic value of her milk, although this would appear to be a subsidiary one.* The following are the findings in support of this theory:

1. During Period II (dry fodder in the dark), the incidence of rickets in rats receiving 3 and 5 cc. doses of the six samples of milk tested was definite and high, and although this was correlated with a high incidence of rickets in the control animals, this indicated milk of low anti-rachitic potency, because the milk-fed animals on these doses showed a higher percentage of rickets than the controls.

2. During Period V (dry fodder in the light), when the cow was out of doors or exposed to light of low intensity, the anti-rachitic value of her milk was at a higher level than that tested during Period II. 3 cc. of these milk samples were protective.

3. The histological evidence given above is supported by results obtained from the calcium determinations of the bones of the same rats. With milk from Period II, the deposition of calcium in the bones was at a slightly, but consistently, lower level than that obtained with similar doses of milk from Period V (see Figs. 1 and 2).

The anti-rachitic potency of the milk during the period of pasture-feeding (Period IV) was, however, much greater than that yielded in Period V. The lift in the curve obtained with 3 and 5 cc. of milk [see Luce, 1924, fig. 2, p. 727], the level of the percentage increase in calcification over controls (Fig. 2, Curve IV) and, moreover, the high protective value of low doses of pasture milk shown in rats on McCollum's No. 3143 diet [Luce, 1924], give striking evidence of this.

The diet of the cow therefore appears to be the main factor in determining the anti-rachitic value of her milk, for with a diet deficient in fat-soluble vitamins it has been found impossible, by the action of sunlight alone, to raise materially the anti-rachitic value of her milk.

Hume [1923] has shown that there is an interaction between vitamin A and light, the latter causing an increased growth in rats maintained upon a diet in which this vitamin is lacking, but concludes that this interaction does not involve a photosynthesis of the vitamin but a mobilisation or economy of a store already existing in the animal's body.

Steenbock and Nelson [1923] suggest that light is without effect on *vitamin A* whilst potent to liberate a reserve of the *anti-rachitic organic factor*, and that the increased growth obtained in Hume's experiments under the influence of irradiation, is due rather to the action of light upon an *anti-rachitic* than a *growth-promoting* factor (vitamin A). In support of this theory, the results of the present experiment indicate that insolation of the cow has some effect in maintaining a certain level of anti-rachitic potency in milk, and little or no effect upon its power to promote growth. The converse has also been shown, for when the cow was fed upon fresh green food in the absence of light, an increased growth-value of the milk was observed, without a corresponding improvement in its anti-rachitic value [see Luce, 1924, p. 724]. It appears from this that *two factors are present in milk, that both are influenced by diet, and the anti-rachitic factor also, to some extent, by light.*

Conclusions.

In the original paper, of which this is an extension, it was concluded that the diet of a cow was the principal factor determining both the growth-promoting value and the anti-rachitic value of her milk. The experiment did not permit any conclusion to be drawn as to whether the light impinging upon the cow also contributed to a minor degree.

These further observations show that sunlight, of summer intensity in this country, has no significant effect in raising the growth-promoting value of cow's milk, when the diet of the cow is deficient in fat-soluble vitamins. The degree of exposure to sunlight appears, however, to have a small effect in determining the anti-rachitic value of the milk given.

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CLXIX. OBSERVATIONS ON THE BEHAVIOUR OF A SUBSTANCE GIVING THE NITROPRUSSIDE REACTION IN SKIN AND IN HAIR.

By MADGE KAYE.

(*Received October 20th, 1924.*)

In a preliminary communication to the Physiological Society, Walker [1924] has shown that a compound is present in skin which gives the well-known nitroprusside reaction for glutathione described by Hopkins [1921]. In view of this work it would appear of some interest to detail the results of experiments on somewhat similar lines carried out by the author during the last few months.

Walker does not consider the compound in skin giving this colour reaction to be glutathione. Other substances such as cysteïne and proteins containing –SH groups also give this or a similar colour with sodium nitroprusside. Cysteïne, however, has never been shown to be present in tissues to a sufficient extent to give the strong reactions observed, and proteins containing –SH groups would not be washed out with water from the cells in skin. The substance in skin which gives the reaction does wash out with water, and therefore cannot be a protein. On the other hand, Hopkins has isolated glutathione from muscle cells and observed it in practically every known tissue. The compound in skin is found in the erector pili muscles and is also associated in other parts of the skin with cells or living tissue, the reaction being particularly strong where metabolism is intense. Hopkins, in his original paper, suggested that glutathione is associated with active metabolism. Collagen and elastin fibres, the stratum corneum, hair shafts and other structures of extra-cellular origin do not give the reaction. It is significant that these structures are not in themselves living tissues but are only the products of active cells. It has, therefore, been assumed in this paper that the substance in skin which gives the nitroprusside reaction is glutathione.

The technique employed was histo-chemical, and essentially the same as that described by Walker. The sections, however, were about 20–30μ in thickness, cut by a freezing microtome, as it was found to be much easier to localise the less intense reactions in definite regions of the skin in the thinner sections.

The skin of ox and calf and also hairs plucked from living subjects have been examined.

The parts of ox and calf skin which give the nitroprusside reaction and the intensity of this in each case are shown in Table I.

Table I.

Parts of skin	Intensity of reaction
The epidermis (except the stratum corneum)	Intense colour (magenta)
All cellular layers of hair follicles	Intense colour
Roots of young hairs	" "
Roots of mature hairs	" "
Portion of hair just above the root	" "
Walls of arteries	Pale pink colour: not intense magenta
Walls of veins	" " "
Erector pili muscle	" " "
Cells lining sebaceous glands	" " "
Cells lining sweat glands	" " "
Groups of cells in the dermis (papillary layer)	" " "

It will be seen that the intense coloration is only found in certain structures. It is very fleeting, fades to pink and finally disappears. Other structures only show the pink colour.

The substance giving the reaction is stable for some time after death, and a good reaction is obtained up to three or four days after the skin has been removed from the animal.

Almost the whole of the compound is washed out of the tissues by running water within twenty-four hours, but if the skin is soaked in still water for the same length of time the tissues give a coloration almost as intense as that given by the fresh skin. After sixty-eight hours in still water, only those parts of the skin giving a strong coloration in the fresh condition show any reaction at all, and, with the exception of the portion of the hair just above the root which gives a strong coloration, these parts only show a pale pink colour with the nitroprusside reaction.

During drying, the substance is almost completely oxidised, and sections of dried skin examined after softening in water show only a slight coloration just above the hair root unless treated with a reducing agent. Zinc and dilute hydrochloric acid were used, sodium chloride being added to prevent excessive swelling of the skin in acid. After reducing, the coloration of the hair root and of the shaft just above the root is greatly intensified, and slight coloration also develops in the epidermis. It is obvious, therefore, that the substance in the oxidised form survives drying and can be detected by the nitroprusside reaction after being converted into the reduced form.

The substance is also stable to some degree against alkalies (lime water and ammonia), acid (hydrochloric acid) and sodium chloride.

Table II shows the coloration produced in skin with the nitroprusside reaction after previous treatment with the above reagents.

It can be seen from Table II that the position in the skin where the glutathione reaction can be demonstrated longest is in the portion of the hair shaft just above the hair root. This is probably due to the keratinous layer of the hair checking penetration.

Table II.

Reagent	Time	Colour reaction: Epidermis	Hair follicles	Hair roots	Hair shafts just above roots
Saturated lime water	14 days	0	0	0	+*
Ammonia *N*/20	24 hours	+†	+†	+++*	+++*
Hydrochloric acid *N*/100	24 ,,	+	+	+++	+++
Sodium chloride sprinkled on skin	24 ,,	0	0	+	+++

* Before and after reduction. † After reduction.

(+) = Very faint or doubtful reaction.
\+ = Weak reaction.
++ = Fairly strong reaction.
+++ = Strong reaction.
++++ = Intense reaction.

The variation in the amount of the substance present or active in living hairs has been determined by this colour reaction. Human hairs, both strong and weak, were taken from a number of subjects. In each case the root bulb was surrounded by cellular tissue of the hair follicle which adhered to the root as the hair was plucked out. The reaction occurs in two stages:

(1) Firstly in the adhering cells around the hair root (these are the first to be penetrated by the solutions).

(2) Later in the hair root itself and the small portion just above the root. The root and hair are penetrated a little later than the follicle cells.

The two stages are quite definite. The coloration in the cells of the follicle of both weak and strong hairs is usually intense, but it is fleeting and fades rapidly. The coloration in the hair root and just above it varies considerably with the condition of the hair; it develops more slowly than that of the follicle cells, but is more permanent. In strong, healthy and pigmented hairs, the coloration of both the follicle and hair root is intense. In grey or white but apparently vigorous hairs, *i.e.* the root bulbs of which are large and well-shaped, the coloration in the follicle is intense, but in the hair root is not so strong as in the pigmented hairs. In grey or white hairs which are obviously weak or the roots of which are degenerating the coloration in the follicle is intense, but in the hair root it is either faint or absent.

It would appear from this that the reaction is intense where there is active metabolism, but where the metabolism may be considered to be reduced, the reaction is correspondingly weaker.

At the time that these experiments were being carried out, some rats which had been fed on experimental diets were given to the author by Miss Boas and Miss Luce. The rats supplied by Miss Luce [1924] were fed on a casein diet and the condition of the hair in some was normal, whilst others showed loss of the long hairs and patches of baldness covered only with weak downy hairs.

The rats supplied by Miss Boas [1924] were fed on an egg white diet, and were in a much poorer condition than those fed on the casein diet. The animals showed very poor growth of long hairs, and an almost universal development

of baldness except for the weak downy hairs. Bran was included in the diet of some of these rats, but not in that of all. A distinct development of scurf and a thickening of the stratum corneum was noticeable, but this was far greater in the case of those rats from whose diet bran was excluded.

Hairs taken from all these animals were examined for the nitroprusside reaction.

The results are given in Table III.

Table III.

A. *Normal rats on casein diet.*

	Reaction			
	Long white hairs	Long black hairs	Short white hairs*	Short black hairs*
Reaction given by hair follicles	+ + +	+ + +	+ +	+ +
Reaction given by hair roots	+ + +	+ + +†	+ +	+ + +

B. *Rats fed on casein diet, showing loss of hair and development of bald patches.*

Reaction given by hair follicles	+ +	+ + +	0	0
Reaction given by hair roots	+ +	+ + +	0	(+)

C. *Rats fed on dried egg white diet + bran.*

Reaction given by hair follicles	+ +	+ +	0	0
Reaction given by hair roots	+	+ +	0	(+)

D. *Rats fed on dried egg white diet and no bran.*

Reaction given by hair follicles	+ +	+ +	0	0
Reaction given by hair roots	+	+	0	(+)

* In C and D taken from bald patches. † Stronger than in white hairs.

In sections of skin from C and D the reaction given by the various structures was as follows:

	C	D
Hair roots of long hairs	++	+
„ „ short „	0	0
Hair follicles	++	++
Epidermis except stratum corneum	++	(+)

The stratum corneum in C was little thicker than in the normal rat; in D it was enormously thickened.

The reason for the shedding of hair in animals fed on these diets is not yet known. It is quite evident, however, that little or no substance giving the nitroprusside reaction of glutathione is present in the hairs which have a very low metabolism.

Again, the substance is present or active on the whole to a greater degree in pigmented than in white hairs.

From the reactions given by the epidermis in the two cases of scurfy skin, it is suggested that in excessive or pathological metabolism the substance may only be present, or at any rate active, to a very reduced degree.

SUMMARY.

1. The substance which gives the nitroprusside reaction of glutathione is present in the cellular structures of the skin, *e.g.* in parts of the epidermis and hairs, in the hair follicles, the lining of the sebaceous and sweat glands, the erector pili muscle, and in the walls of arteries and veins.

2. It is not present or active to the same degree in all these tissues, and is absent in structures of extra-cellular origin such as collagen fibres, hair shafts, stratum corneum, and elastin fibres.

3. It is present to a larger extent in the roots of healthy pigmented hairs than in those of healthy white hairs, and it decreases in quantity or activity as the vitality of the hair decreases.

4. The substance is intimately connected with active metabolism and varies with the variations in metabolism.

In conclusion, the author wishes to extend her thanks to the Director (Dr R. H. Pickard) and Council of the British Leather Manufacturers' Research Association for permission to publish this work.

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CLXX. ON THE INFLUENCE OF THE HYDROGEN ION CONCENTRATION UPON THE OXYGEN CONSUMPTION IN SEA-WATER FISHES.

By JAYME REGALLO PEREIRA.

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(*Received October 22nd, 1924.*)

The oxygen consumption of fishes has been studied by several workers but, so far as I know, the only workers who have attempted to observe the effect of varying the conditions of the water are Gardner and King [1923], who observed the influence of temperature on the oxygen consumption in fresh-water fishes. Variation in temperature, however, is not the only abnormal condition which can occur, and this paper deals with experiments on the influence of the hydrogen ion concentration upon oxygen consumption in sea-water fishes.

The estimation of the oxygen was made by using the titration method of Winkler [1888, 1889] as modified by Birge and Juday [1911]. This method has given very reliable results in the hands of different investigators [see Butler and Coste, 1923; Lund, 1918; McClendon, 1917; Chlopin, 1896, and Amberson with his associates, 1924]. The fishes used in my experiments were *Fundulus heteroclitus*, which were placed in large reservoirs containing 10 litres of fresh sea-water. On the top of the water a layer of half a centimetre of paraffin oil was placed in order to avoid direct contact of air and continuous solution of oxygen in the water. Samples were collected each 10 or 15 minutes in the manner already described in the paper of Amberson and his associates. Different numbers of fishes were used in each reservoir, and their weight was carefully determined before and after the experiments. The experiments were carried out until the dissolved oxygen was reduced to one-tenth. Two reservoirs were employed containing sea-water of different p_H. In order to check the results, in some experiments the groups of fishes used in the two reservoirs were interchanged and the same p_H again tested after a suitable interval, during which the fishes were allowed to recover in fresh sea-water.

The fact that organic matter as well as bacteria, introduced into the water by the fish, consumes oxygen, has been invoked as a serious error in the use of Winkler's method. Gardner and Leetham [1914], however, have shown that this assumption does not hold. On the other hand, the objection that the carbon dioxide produced by the fish may increase the acidity of the water to a higher degree than that chosen for experiment, has also no value; Amberson

and his associates say that the production of carbon dioxide only causes a slight change in the hydrogen ion concentration of the water (from p_H 8·1 to 7·6) when the oxygen tension of the water is reduced to 10 %. Further, they found that "changes in CO_2 tensions much exceeding this amount have no effect upon the rate of oxygen consumption." Titration of the hydrogen ion concentration of the sea-water was made by using the colorimetric method, and the increase in the acidity of the water by adding the necessary number of drops of a $N/10$ hydrochloric acid solution, according to the following table:

p_H	$N/10$ HCl to 100 cc. (drops)
8·0	0
7·0	12
6·0	33
5·0	57
4·0	65

The irritant action of an acid medium on the skin, bringing about a possible muscular activity of the fish, led me to anticipate a greater oxygen consumption. Such an assumption appeared not to be true, for I observed in thirteen experiments that in sea-water of various hydrogen ion concentrations, in general, the more acid the medium the less is the oxygen consumption per g. of fish per litre of water per hour. This was tested between p_H 8·0 and 4·0 as follows:

p_H	Temperature ° C.	Total weight of the fish (in g.)	O_2 used per gram per litre per hour	Average
8	22·0	139	·018724	
8	22·0	153	·016220	
8	21·0	189	·017070	·017338
7	22·0	167	·016838	
7	20·5	282	·016186	·016512
6	22·0	170	·014626	
6	21·0	130	·017600	
6	21·5	211	·015180	·015802
5	22·0	352	·010280	
5	20·5	293	·013160	·011720
4	22·0	193	·013804	
4	22·0	295	·010170	
4	21·0	237	·012320	·012098

The oxygen consumption is seen to be a minimum at p_H 5.

Two further experiments were made on the alkaline side of neutrality. With p_H 9·0 a consumption of 0·18006 was observed, and with p_H 10·0 of 0·017580. These results seem to indicate that there is an optimal hydrogen ion concentration which can be located around p_H 8·0, which is the reaction of the normal sea-water. As was expected, a greater activity of the fish was observed in a more acid medium. On the other hand, I found that the fish remained for a long time with their mouths closed, thus avoiding contact of the irritant water with their gills. The diminution of the respiratory movements corresponded with the lowered oxygen consumption when the acidity of the medium was increased.

The low oxygen tension in the water at the end of the experiments only once caused the death of a fish. On the other hand, *fainting* was observed in some fishes when approaching the end of the experiment; in such an abnormal state, however, they did not stop breathing and recovered completely in fresh sea-water.

This work was carried out in the Laboratory of Physiology of the Marine Biological Laboratory, at Wood's Hole, Mass. I am indebted to S. Culver Williams, who assisted me in the course of the experiments.

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CLXXI. NOTE ON THE EFFECT OF AMMONIUM SULPHATE AND OTHER SALTS ON THE COLORIMETRIC ESTIMATION OF PHOSPHORUS.

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(*Received October 27th, 1924.*)

In view of the present extensive use of the Briggs colorimetric method for the estimation of phosphorus, certain difficulties which have been encountered in the application of this method to solutions containing high concentrations of salts are worthy of record.

Effect of Ammonium Sulphate.

During the course of investigations at present in progress, it was desired to estimate, by the Briggs [1922] method, the phosphorus content of small quantities of solutions containing proteins. For the removal of the latter, half saturation with ammonium sulphate was employed, but the results were quite irregular, in nearly all cases low values being obtained. The cause of the irregularity was the high concentration of ammonium sulphate. This salt, in concentrations above 1·4*N*, markedly decreases the velocity of colour production in solutions containing the amounts of phosphorus which are those normally estimated by this process.

The effect of measured amounts of ammonium sulphate in solutions in which definite quantities of phosphorus were present was investigated. After the addition of Briggs' reagents in the usual way, the colour produced in these solutions at the end of half-an-hour was compared with a standard similarly treated, but without the addition of the ammonium sulphate. Plotting these half-hour values, calculated as alleged phosphorus content (= depth of colour relative to standard) against the concentration of ammonium sulphate, the curve of Fig. 1 is obtained.

It is at once evident that the colour is markedly depressed as the concentration of ammonium sulphate increases, although when below a certain value —in this series, 1·4*N*—the colour attained in half-an-hour is actually deepened by the addition of the ammonium salt. This may be due to the lavender-blue tint which is seen in all solutions containing ammonium salts in more than minimal quantities when treated according to the Briggs technique.

There is, however, no error produced by the small amounts of ammonium sulphate that may result from the micro-incineration method usually employed for determining the total quantity of phosphorus in presence of small quantities of protein. Taking caseinogen as containing 0·8 % P and 16 % N, and supposing quantities are chosen so that 0·05 mg. of phosphorus is present in a total vol. of 25 cc. when the colorimetric determination is made, the concentration of ammonium sulphate derived from the nitrogen of the protein molecule will not exceed 0·003*N*, a figure considerably lower than that found by experiment to be the least affecting the determination. No less than 2 g. of protein need to be oxidised for the ammonium sulphate produced to interfere seriously with colour production.

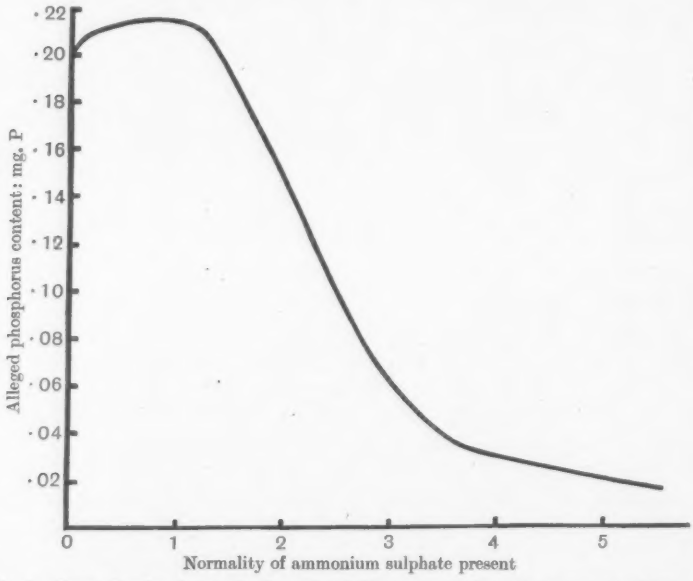

Fig. 1. Curve showing depth of colour after half-an-hour with constant amount of PO_4 and different concentrations of ammonium sulphate. (0·2 mg. P in 50 cc. total volume.)

Although the rate of colour production in presence of ammonium sulphate is slowed, the final depth of colour attained after standing for a lengthy period is practically unaffected. This is clearly shown in Fig. 2 which explains itself.

It will be seen that with a concentration of 1·4*N* ammonium sulphate, the colour production at the end of half-an-hour is only about 80 % of that which the amount of phosphate present should produce.

Since, however, the colorimetric estimation is made against a standard which is itself slowly changing, the curves in Fig. 2 will be somewhat distorted. Such distortion, however, is confined to the readings made within the first

quarter of an hour, since the standard solution has developed at least 93 % of its colour value at the end of half-an-hour.

In view of the observation of Briggs that sulphuric acid when present in excess exerts an inhibiting action upon colour formation, it was suspected that the retardation produced by ammonium sulphate might in reality be due to the sulphate ion. That this is not so is evidenced by the fact that neither magnesium nor zinc sulphate has any disturbing effect, although sodium sulphate depresses colour formation to a slight extent. Neither can the effect be due to the ammonium ion, for solutions treated with ammonium nitrate exhibit no irregularity.

Since the diminution in rate of colour production is not due to the NH_4 ion, nor to the SO_4 ion, it is possible that it may be the result of some specific effect of un-ionised ammonium sulphate.

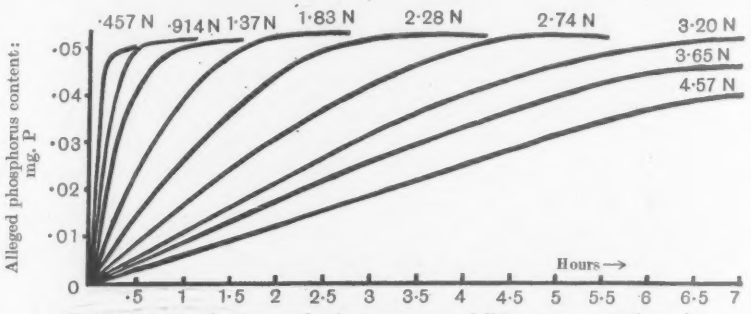

Fig. 2. Velocity of colour production in presence of different concentrations of ammonium sulphate. (0·05 mg. P in 25 cc. total volume.)

It has been found that ammonium sulphate is without effect once the colour has developed ($\frac{1}{2}$ or 1 hr.); hence the inhibition is upon the *progress* of the reaction and is not merely a disturbance of the ionic equilibrium of the solution. Neither can it be due to an interference with the preliminary reaction—

Ammonium Molybdate + Phosphate → Ammonium Phosphomolybdate

for maintenance of the two reactants at a temperature of 65° for one hour before the ammonium sulphate is added in no way influences the retarding effect of the latter.

Effect of Anticoagulants.

One of the disadvantages attending the use of the Bell-Doisy [1920] technique was that when applied to plasmas containing excess of anticoagulant, the colour production was interfered with. Denis and von Meysenbug [1922] investigated the case of sodium citrate and potassium oxalate with reference to this method.

In his first paper Briggs [1922] points out that his technique is not affected by the concentrations of these salts usually employed.

The present author has found that ammonium oxalate, sodium oxalate, sodium sulphate, sodium fluoride and sodium citrate all interfere with colour production if their concentrations be sufficiently great. The limiting concentrations at which interference first occurs have been determined for these salts and are given below.

Concentration of Phosphorus—0·05 *mg. in* 25 *cc.*

Anticoagulant	Concentration at which interference occurs	Approximate concentration usually present
Sodium oxalate	0·008*N*	0·002*N*
„ citrate	0·008*N*	0·003*N*
„ sulphate	0·8*N*	0·109*N*
„ fluoride	0·01*N*	0·007*N*

Following the usual practice of adding nine parts of blood to one part of sodium fluoride (3 % solution) and then using the quantities recommended by Briggs [1924], the concentration of fluoride in the final solution will not exceed 0·007*N*; thus no interference will result. The concentration is, however, rather dangerously near the strength at which interference first occurs.

It will be apparent that although the colorimetric method is eminently suited for the estimation of small quantities of phosphorus, there are causes of error which unless guarded against are liable to render the results inaccurate.

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CLXXII. AN APPARATUS FOR THE GRAPHIC RECORDING OF OXYGEN CONSUMPTION AND CARBON DIOXIDE OUTPUT, ESPECIALLY ADAPTED FOR CLINICAL WORK.

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From the Laboratory of Zoophysiology of the University of Copenhagen.

(*Received October 27th, 1924.*)

Experience with Krogh's [1923] recording respiration apparatus has shown clearly that it is a very great advantage in clinical work to have oxygen consumption graphically recorded, because the graph makes it possible to see whether the respiration and metabolism are uniform or not, a control which is specially necessary in studying subjects untrained in metabolic experiments, such as most patients will be.

While the determination of a patient's oxygen consumption is relatively easy to obtain, there are certain special difficulties in determining the carbon dioxide, arising from the fact that a slight change in the ventilation may give rise to a considerable change in the carbon dioxide content of the body; therefore metabolic experiments of short duration with patients often give very misleading results regarding the respiratory quotient, as patients still more than other untrained persons tend to alter their ventilation when breathing through a mask or mouthpiece.

An apparatus which could record not only the oxygen consumption but also the carbon dioxide output would be helpful in clinical metabolic work, because it would permit the observation during the experiment of every change in ventilation, oxygen consumption and carbon dioxide output, and therefore give much safer results than an ordinary apparatus.

Under many circumstances it would also be convenient to avoid gas analyses.

In the following pages an apparatus of this type will be described.

Hanriot and Richet [1891] have invented and used an apparatus for measuring both oxygen consumption and carbon dioxide output; they use three equal gas meters; one measures the inspired air, the second the expired air, and a third the expired air after absorption of the carbon dioxide in a suitable absorber. The difference in reading between the second and the third meter shows the amount of carbon dioxide eliminated during a certain time, while the difference between the first and the third shows the oxygen absorbed. The method of Hanriot and Richet has not been generally adopted, undoubtedly because there are special sources of error in gas meters which are difficult or even impossible to overcome with the arrangement described.

Closer investigations have led the writer to the opinion, that the most serious errors arise from changes in the air pressure affecting the water level. An example will show the importance of this. An air current is led through a gas meter at a constant rate, the volume of air per revolution being measured independently of the meter; if now a sort of braking effect is exerted on the shaft of the gas meter the result will be, first that the air pressure just great enough to run the drum will increase, and therefore the water level in the drum will be changed, and hence the volume of air corresponding to the revolution of the meter will also be considerably changed.

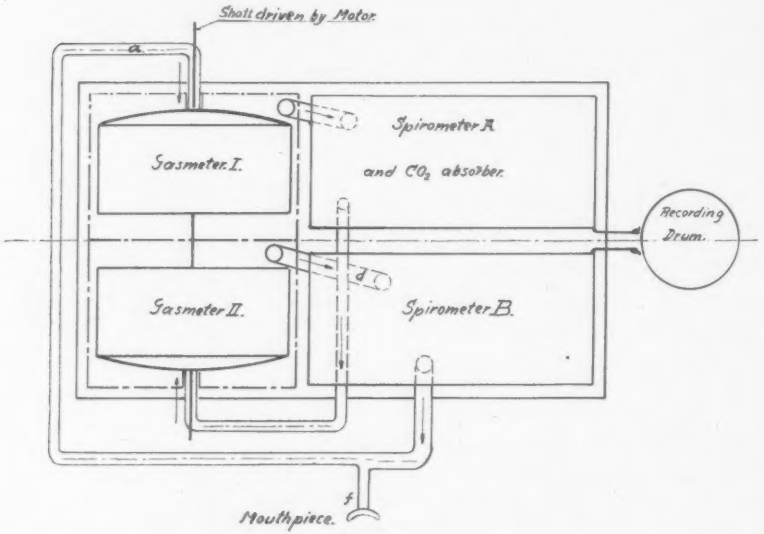

Fig. 1.

Fig. 1 shows an arrangement partly based on the principle of Hanriot and Richet, in which the sources of errors arising from the gas meters have been reduced to such an extent that accurate determination of the human respiratory exchange can be made.

The apparatus is of the closed circuit type, the air being circulated by two symmetrical gas meters, arranged on a common shaft, which is revolved at a suitable rate (one to three revolutions per minute) by a small electric motor fitted with a simple governor to secure constancy of the rate. The gas meter drums are placed in a common water-bath, and as this water-bath is mounted on levelling screws it is easy to obtain such an angle between the axis of the double gas meters and the water level that the two parts of the gas meters are giving exactly the same volume of air for a full revolution. (If the volumes of the drums are exactly identical the axis of course has to be parallel to the water level.)

Gas meter I pumps air at a certain rate from the pipe (*a*) to the recording spirometer (*A*), which is of the type described by Krogh and used in his recording respiration apparatus. The box of the spirometer is filled with moist soda lime to absorb CO_2, and the pressure of the air in it remains constant whatever the position of the spirometer bell. Gas meter II pumps just that quantity of air out from spirometer (*A*) which was put in by meter I and delivers it to the pipe (*d*). When therefore the ingoing air contains CO_2 the quantity of this gas will be recorded by the downward movement of the spirometer bell (*A*).

The pipe (*d*) carries the air through a second recording spirometer (*B*) back to meter I, but at (*f*) the pipe is provided with a side tube which can be connected through a mouthpiece or mask with the respiratory system of a patient.

If this side tube is closed and the gas meter motor and recording drums started each spirometer will write a wave line, each wave corresponding to one revolution of the double gas meter. The waves arise from small irregularities, *e.g.* eccentricity in the two gas meters' drums, but if the levelling screws of the water-bath are correctly adjusted the wave lines will be horizontal.

If a person is breathing into the side tube the spirometer (*B*) will record the respirations of the subject, but the graph will show a slight decline corresponding to the difference between the volume of air inspired and expired. It is well known that this difference is due to the volume of CO_2 eliminated being (as a rule) smaller than the volume of O_2 absorbed, and if the decline of the graph is measured by means of a scale divided in litres over a period of, say, 10 minutes the resulting figure represents the oxygen absorbed – the CO_2 eliminated during that period. Simultaneously spirometer *A* records directly the CO_2 output, and the oxygen consumption is therefore easily calculated: oxygen consumption = carbon dioxide output + difference between oxygen consumption and carbon dioxide output.

The air current in the apparatus is running so fast that the volume passing through a transverse section in one minute is not less than thrice the ventilation of the subject in the same time; if this condition is maintained valves are superfluous.

Fig. 2 shows the apparatus in a convenient form which has been in use for about two years with entire satisfaction. The gas meters and spirometers are arranged in a common water-bath, the spirometers recording on a common drum, most conveniently with ink of different colours. The water-bath is fitted with an overflow tube to secure constant water level; it contains about 100 kg. water, and the expired air is led through a pipe of considerable length which is immersed in the bath to cool the air to the temperature of the bath before it enters the gas meter. From the service pipe to the spirometer (*B*) there is a wide pipe to secure that every change in air pressure caused by the respiration is taken up promptly by the spirometer (*B*), so that there can be an absolutely constant pressure in the gas meter I.

The accuracy of the results largely depends upon the care with which the spirometers are balanced; every change in the air pressure in one of the spirometers will affect the water level in the corresponding gas meter and so disturb its accuracy. The spirometers therefore are arranged on special bearings and balanced with a double set of counterbalances, a large one for gross and a small one for fine adjustment. The counterbalances are adjustable horizontally and vertically, this allowing convenient compensation for buoyancy of the spirometer.

The accuracy of the measurements is greatly increased by dissolving 1 litre glycerol and 5 g. sodium glycocholate in the water in the bath, this securing a homogeneous film of liquid on the spirometer bells and gas meter drums, when these parts are moving from the water into the air; this liquid also gives only a relatively small resistance when a dry spirometer bell is dipping in it. To secure that the liquid is slightly acid a small amount of salicylic acid may be useful.

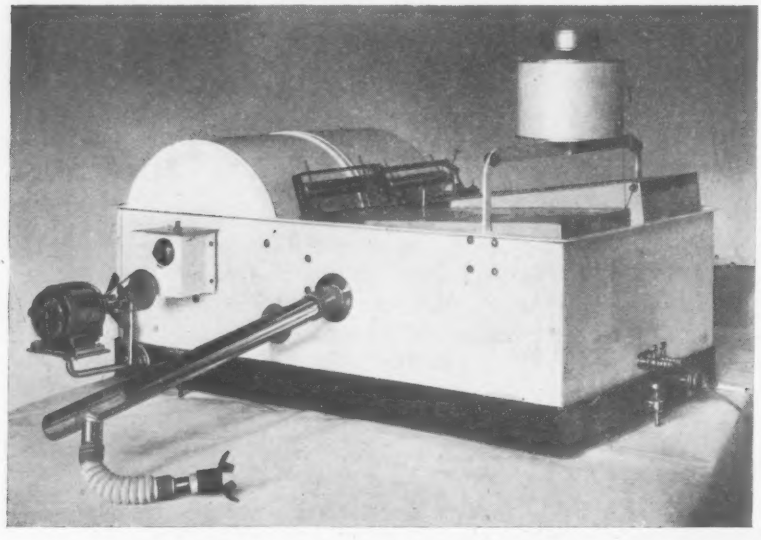

Fig. 2.

The apparatus is tested for tightness by placing a weight of 100 g. on the spirometers and recording, without running the meters; if the apparatus is tight the lines written will be horizontal.

Then it is adjusted with the meters running, by means of the screws, so that the two gas meters give the same volume of air per revolution; the spirometers will in such case record horizontally.

The balancing of the spirometers is correct, when the wave lines are horizontal, whether the spirometers are filled with air or nearly empty; this

securing that the buoyancy is correctly compensated. It is of course the safest plan to let the apparatus record for some time before or after each experiment, to be sure that it is correctly adjusted. If the wave lines are not quite horizontal, it is not necessary to adjust the apparatus. The change in level can be measured and allowed for in calculating the result. If, for instance, spirometer *A* rises during a complete revolution 0·15 litre, the position of spirometer *B* will (when the apparatus is closed and air-tight) be lowered to the same extent, as shown in Fig. 3. A corresponding correction must therefore be added to the recorded volume of CO_2 and subtracted from the record of the difference $O_2 - CO_2$.

Special tests have been made to make sure that the respiratory movements and the high temperature of the expired air cannot influence the records. On the side tube was attached a large rubber ball which was forcibly compressed, corresponding to a frequent deep breathing. The recorded line was parallel to the wave line recorded, when the rubber ball was not in use. In another experiment the rubber ball was inserted on a tin spiral tube immersed in very hot water to secure that the temperature of the expired air had no disturbing influence; the line of "respirations" recorded was parallel.

Finally several experiments were made by burning alcohol in the apparatus. The side tube was removed and the air current conducted through a suitable container enclosing a small alcohol-burner, which was supplied with alcohol from a burette. At the moment when the alcohol level passes a division on the burette, the pens are moved from the recording drum for a few seconds, and so a convenient record of the rate of combustion of the alcohol can be obtained. The table gives a series of such experiments:

No.	T.	Bar.				R.Q.	Oxygen 0° 760 mm. (litres)
			Spirometer	*B*	160		
1	17°	763	„	*A*	317	·665	4·42
					477		
			„	*B*	161		
2	17°	763	„	*A*	318	·664	4·44
					479		
			„	*B*	163		
3	19°	763	„	*A*	321	·663	4·46
					484		
			„	*B*	162		
4	19°	763	„	*A*	319	·662	4·42
					481		
			„	*B*	162		
5	19°	763	„	*A*	323	·666	4·46
					485		
			„	*B*	162		
6	19°	763	„	*A*	325	·667	4·48
					487		
					Mean	·664	4·45

In each experiment 3·10 g. alcohol $\left(d\,\frac{15°}{15°} = 0{\cdot}7970\right)$ were burned corresponding to $\frac{310 \times 99{\cdot}1 \times 67{\cdot}23}{46} = 4{\cdot}49$ litres oxygen.

Theoretical oxygen consumption 4·49 litres.

Theoretical R.Q. 0·667.

The table shows the great uniformity of the measurements, and the agreement between the measured and theoretical quotients is also very good. There is a difference of about 1 % between the calculated and measured oxygen consumption, but it is doubtful whether this is due to an error of the measurement with the apparatus or to an error in the determination of the amount of alcohol burned.

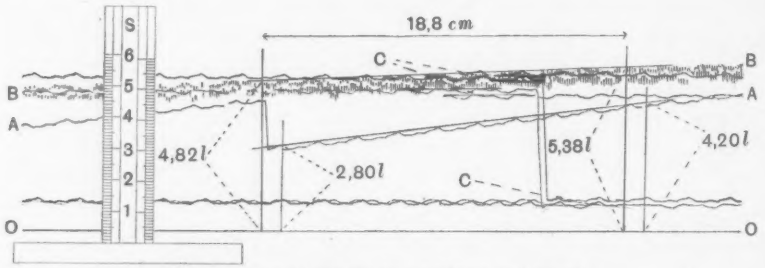

Fig. 3. Curves of respiration experiment and control.

S. Special scale for measuring curves divided in litres; right for measuring CO_2 (spirometer *A*), left for measuring differences $O_2 - CO_2$ (spirometer *B*).

O—O. Zero line for measurements.

C. Control curves drawn by spirometers. Heavy line spirometer *A*, faint line spirometer *B*. The lines are seen to be nearly horizontal. Two controls are made, one with *A* in top position and *B* nearly empty, another with positions reversed. The error per revolution is in both positions and for both spirometers the same (0·15 litre), showing correct compensation for buoyancy.

A. Curve from experiment. Spirometer *A* showing CO_2 absorption in 10 min. (distance 18·8 cm.).

B. Curve from experiment. Spirometer *B* showing respiration of subject and difference $O_2 - CO_2$ in 10 minutes. For calculation of results see below.

Fig. 3 shows the graph of an experiment and the special scale used for the measurements; the readings in litres are written on the paper and the calculation is as follows:

T. 18°, Bar. 752 (reduction factor 0·909).

Correction:	Litres
for a total revolution of the drum (30 minutes)	0·15
for 10 minutes	0·05

	Litres
CO_2 before experiment	4·20
CO_2 after experiment	2·80
Difference	1·40
+ correction	0·05
CO_2 output	1·45

$O_2 - CO_2 = 0{\cdot}51$
$CO_2 = 1{\cdot}45$
O_2 1·96 R.Q. = 0·740
O_2 reduced to 0° 760 and
$0{\cdot}909 \times 1{\cdot}96 = 1{\cdot}78$ litres
CO_2 reduced to $0{\cdot}909 \times 1{\cdot}45 = 1{\cdot}32$ litres

	Litres
$O_2 - CO_2$ before experiment ...	5·38
$O_2 - CO_2$ after experiment... ...	4·82
Difference	0·56
− correction	0·05
Difference between O_2 consumption and CO_2 output	0·51

The heat production can be calculated with the help of tables compiled by Zuntz and Schumburg [1901].

The calculation and tables are also given by Krogh [1916].

The ventilation can be calculated in the following manner. With a good planimeter the total area between inspirations and expirations in an experiment is measured; this is easily obtained by moving the pin of the planimeter from top to top of all the expirations in the experiment, following the last respiration down to the inspiration and then through all the deepest points of the inspirations and up to the point in which the measuring has started; the reading of the planimeter will then give an area, and this area divided by the length of the experiment in centimetres on the abscissa will give the mean depth of respiration in centimetres; which can be transformed into litres by means of the special scale. The average respiration multiplied by the number of respirations during the experiment and divided by the duration of the experiment in minutes will give the ventilation per minute.

I wish to express my most sincere thanks to Prof. A. Krogh for much helpful advice, and I am also indebted to Mr J. Jensen for special technical assistance.

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CLXXIII. FURTHER EXPERIMENTS ON THE INFLUENCE OF THE PARENTS' DIET UPON THE YOUNG.

I. THE INFLUENCE OF THE FATHER'S DIET.

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(*Received October 27th, 1924.*)

In our previous experiments [Korenchevsky and Carr, 1923] we studied the influence of the father's diet before and after conception upon the number, weight and chemical composition of the young *at birth*. The results obtained showed that feeding the father before conception on a diet deficient in fat-soluble factor had no influence whatever on the young, either on their number or weight or the water, calcium, phosphorus and nitrogen content of their bodies.

In the present investigation we have tried to discover whether the father's diet before conception has any influence upon his productiveness and upon the vitality of the young and the calcification of their skeleton at the day of *weaning and later*.

Experiments were conducted in the following way on 36 females and 14 males. A group of adult females from the same litter were taken when about two to three months old and were fed on a normal diet *NB* (see composition below) for at least one month before the experiment began. The males were chosen from another litter, half of them were kept on a normal diet *N* 2, which was very rich in fat-soluble factor (see below) and contained ample calcium. The other half of the males were kept on the same *NB* diet as the females, containing butter only as the source of the fat-soluble factor and not cod-liver oil as is the case in diet *N* 2 and also containing sufficient calcium although only half as much as is in diet *N* 2. For two weeks before conception the males, which had been kept on this *NB* diet, were fed instead with diet $-A$, deficient in fat-soluble factor. This was done to reduce the amount of fat-soluble factor in the body of the males without making them sterile. On the day they were mated, the females of each litter were divided into two groups, one group were put with the males on *N* 2 diet, the other with the males on $-A$ diet. In a few cases, females were taken from different litters for the two groups. The same proportion of females and males were mated in each group. One male was put with one to four females. Copulation occurred. After a few

days the males were segregated and the pregnant females were put in separate cages. During the whole of pregnancy the females were kept on the normal *NB* diet and during lactation on $-A$ diet (deficient in fat-soluble factor). After birth all except five young from each litter were destroyed. Two of the young were killed on the day of weaning for chemical and histological examination of the skeleton. The other three continued to be kept on $-A$ diet until they were 65 days old, when they were killed. The technique of the experiment was the same as described in previous papers [Korenchevsky, 1921, 1922]. Here we will only give the composition of the diets used.

All the diets have the following basal formula:

Purified casein ...	18 g.	Orange juice	8 cc.
Starch	52 g.	Distilled water	50 cc.
Fat	15 g.	McCollum's (1917) salt mixture	5 g.
Dried yeast ...	5 g.		

The variations in the diets are clear from Table I.

Table I.

Diets	Kind of fat used in the diet	Amount of calcium %: In fresh paste	Amount of calcium %: In air-dried paste	Remarks
NB	Butter	0·26	0·4	Normal diet, containing an adequate, but not excessive amount of calcium and fat-soluble factor
N 2	80 % butter + 20 % cod-liver oil	0·52	0·8	Normal diet, rich in fat-soluble factor and calcium
$-A$	Cotton-seed oil	0·26	0·4	Diet with an adequate amount of calcium, but deficient in fat-soluble factor

The results of these experiments were as follows:

A. In the group containing fathers on *N* 2 diet (above) before and during mating (mothers fed on *NB* diet before and during pregnancy and $-A$ diet during lactation):

1. From the 24 matings no pregnancy ensued in one case (4·2 %).
2. Of the 23 litters born three were eaten (13·0 %).
3. Out of 75 young left in 15 litters and studied in this group 10 died (13·3 %) before the end of the experiment from different morbid causes (diarrhoea, accidental infections and general weakness).
4. The average weight of a young rat at the day of weaning (24 days old) was 31 g. for male and 29 g. for female; when 65 days old 62 g. and 51 g. respectively.

B. In the group containing fathers on $-A$ diet for two weeks before mating (mothers fed on *NB* diet before and during pregnancy and $-A$ diet during lactation):

1. From 34 matings no pregnancy ensued in eight cases (23·2 %).

2. Of the 26 litters born 11 were eaten (42·3 %).

3. Out of 70 young left in 14 litters and studied in this group, 13 died (18·6 %) before the end of the experiment from the different morbid causes mentioned above.

4. The average weight of a young rat at the day of weaning (24 days old) was 31 g. for male and 30 g. for female; when 65 days old 61 g. and 55 g. respectively.

The results of the chemical investigation of the skeleton of the young kept on a rickets-producing – *A* diet are clear from Tables II and III.

Table II. *Average chemical composition of the skeleton of the young rats kept on a fat-soluble factor deficient* – A *diet, whose fathers were fed on a diet* rich in fat-soluble factor *and calcium* (N 2).

	Killed when 24 days old In bones			Killed when 65 days old In bones		
Experiment no.	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %
1	62·4	4·7	12·5	60·0	5·6	13·9
2	60·0	4·8	12·0	—	—	—
3	58·2	4·8	11·5	—	—	—
4	59·3	4·9	11·8	49·2	6·4	12·5
5	61·8	4·9	12·8	55·1	6·0	13·3
6	62·9	4·9	13·3	55·2	5·2	12·4
7	61·7	4·9	12·7	51·1	7·0	14·2
8	63·6	5·0	13·7	48·0	6·7	12·8
9	58·4	5·0	12·1	52·0	6·6	13·8
10	62·3	5·2	13·8	46·3	6·3	11·8
11	56·9	5·3	12·2	54·1	6·0	13·3
12	63·0	5·3	14·2	49·8	7·8	15·5
13	58·7	5·4	13·0	46·8	6·9	13·0
14	61·1	5·4	13·8	47·5	8·8	16·8
15	58·2	5·7	13·5	45·1	8·0	14·5
Average	60·6	5·1	12·9	50·8	6·7	13·7

Table III. *Average chemical composition of the skeleton of young rats kept on a fat-soluble factor deficient* – A *diet, whose fathers were fed on* – A *diet* poor in fat-soluble factor.

	Killed when 24 days old In bones			Killed when 65 days old In bones		
Experiment no.	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %
16	66·4	3·7	11·0	—	—	—
17	62·7	4·5	11·9	50·9	7·1	14·3
18	59·3	4·6	11·4	—	—	—
19	61·4	4·7	12·1	52·7	6·5	13·7
20	63·4	4·7	12·8	—	—	—
21	60·7	4·8	12·3	51·5	7·0	14·4
22	60·1	4·8	12·0	—	—	—
23	62·2	4·9	13·0	49·7	6·8	13·5
24	61·2	5·0	12·9	—	—	—
25	61·3	5·1	13·1	49·4	5·9	11·7
26	59·4	5·1	12·5	48·7	7·5	14·7
27	59·3	5·1	12·4	—	—	—
28	62·3	5·2	13·4	51·5	7·4	15·3
29	61·4	5·4	13·9	53·8	6·4	13·7
Average	62·2	4·8	12·4	51·0	6·8	13·9

The figures represent an average from each litter of the analyses of the skeletons of two young rats at the day of weaning and of three young rats when 65 days old, if all the rats lived till the end of the experiment. If they did not all survive, averages were made from the decreased number of young rats in each litter which lived till killed for examination. The results are summarised only in general average tables, because no constant or definite difference was found between the calcification of the skeleton of the young begotten by fathers on a good diet and those begotten by fathers on a deficient diet. The average figures (see Tables II and III) are nearly the same in both groups and in most cases are within the usual fluctuations found in the calcification of the skeleton of rats kept on this fat-soluble factor deficient diet.

However, the skeleton in three litters, Nos. 12, 14 and in most of 15, begotten by fathers kept on *N* 2 diet (cod-liver oil), showed an unusually good chemical composition, especially in those rats killed when 65 days old (Table II). But as this change was found in three litters only and because there was no definite difference in the average figures of Tables II and III, no special significance can be ascribed to this increase in the calcification of the skeleton in the above-mentioned litters.

Histological investigation of the skeleton was made only in nine litters altogether, because no differences had been found in the chemical composition. Picture of osteoporosis with slight rickets prevailed. In correspondence with the chemical results there were no essential histological differences between the groups.

It is of interest to mention here the results of a careful investigation by Dr Ougrumov [see Korenchevsky, 1914], because he came to conclusions which showed the importance of the father's nutrition before conception. Ougrumov made his experiments with rabbits. He mated normally fed females with males which had been starved for varying lengths of time. Litters conceived under these conditions, even though born from normal females, possessed much less vitality: they were so weak that they very often died a few minutes after birth. A chemical investigation of these young rabbits just after birth showed that their bodies contained more water and fat and less nitrogen and calcium than those begotten by normally fed fathers.

Summary.

In our few experiments it was found that:

1. If the diet of the males before conception was deficient in fat-soluble factor, a greater percentage of matings was unproductive.

2. The litters, begotten by fathers kept on a diet deficient in fat-soluble factor, seemed to be weaker: in our 49 successful matings a greater percentage of these litters were eaten by the mothers, and more young died from accidental diseases than amongst those begotten by richly-fed fathers.

3. No difference was noticed in the weight of the young in the two groups nor in the calcium composition of their skeletons.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish also to express our sincere gratitude to Professor C. J. Martin for his continuous support and criticism in this investigation.

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CLXXIV. FURTHER EXPERIMENTS ON THE INFLUENCE OF THE PARENTS' DIET UPON THE YOUNG.

II. THE INFLUENCE UPON THE YOUNG OF AN EXCESSIVE AMOUNT OF FAT-SOLUBLE FACTOR AND CALCIUM IN THE MOTHER'S DIET DURING PREGNANCY.

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(*Received October 27th, 1924.*)

In our previous experiments [Korenchevsky, 1921, 1922, 1923; Korenchevsky and Carr, 1923, 1, 2], we have found that the mother's diet has a definite influence on the young. The results obtained may be summarised as follows.

The diet of the mother before conception and during pregnancy influenced the litter *at birth* in some respects: the mothers kept on normal diet, containing an excess of fat-soluble factor and calcium, bore litters larger in number and total weight than those born by mothers fed on usual or fat-soluble deficient diets. The larger total weight of the litters was due to the greater number of offspring in each litter and not to the increased weight of the individuals. The largest number of young were born dead in those litters whose mothers were fed on deficient diets. At the day of birth the H_2O, Ca, P and N content in the young was nearly the same, irrespective of the mother's previous diet. The normal chemical composition shown to exist at birth in foetuses born of mothers kept on a diet deficient in fat-soluble factors is in accord with the fact that the maternal organism will as far as possible yield all the necessary substances to her offspring by the sacrifice of her own tissues. Therefore, during lactation, a rapid exhaustion of fat-soluble factor from the mother's body was observed, when the mother during this period was on a diet deficient in this respect. However, the storage of these substances, though sufficient at birth, certainly did not suffice in the post-natal life of the offspring because they continued to be kept on a fat-soluble factor deficient diet.

A maternal sacrifice of a similar kind caused by a general fasting of the mother during pregnancy has been clearly shown by Rudolsky [1893]. He performed his experiments on a bitch and six rabbits. Nitrogenous and gaseous metabolism were studied in these animals during the whole of the experiment. He reduced their diets to four-fifths in the rabbits and to two-thirds in the

bitch of the normal minimal ration, required for the maintenance of the animals in metabolic equilibrium. Such a reduction of the ration was accompanied by a loss of protein and fat from the animals' tissues. In spite of this, these females, after being mated with normally fed males and kept during pregnancy on the same food régime as before, gave birth to young, the chemical composition of whose bodies was normal at birth. However, when the ration of food given to the mothers was reduced further, poor abnormal young were born. In this case the mothers were so emaciated that they often died, showing a great loss of weight and profound degeneration of their tissues, after giving birth to their young. If the mothers survived, they returned to their previous weight very slowly, if at all, in spite of ample food.

On the basis of the results of our experiments at *the day of weaning and later*, the following conclusions were drawn.

When the mother is fed during *pregnancy* or *lactation* on a diet deficient in fat-soluble factor or calcium there is a marked increase in the disorders of general nutrition and rachitic changes in the skeleton of the offspring kept after weaning on the same rickets-producing diet. Feeding the mother during *lactation* on a diet rich in fat-soluble factor and calcium hinders for a considerable time the disorganisation of general nutrition and the development of obvious rickets in offspring kept on a diet deficient in fat-soluble factor. The results obtained are due to the influence of the mother's diet during pregnancy and lactation upon her nutrition and therefore through the placenta and afterwards through her milk upon the nourishment of the offspring.

In the present investigation we studied the influence upon the young (1) of fat-soluble factor in the mother's diet during pregnancy, and (2) of an excessive amount of calcium in the mother's diet during pregnancy.

(1) *The influence of fat-soluble factor in the mother's diet during pregnancy upon the growth, general nourishment and skeleton of the young rats.*

In our previous paper [1923, 2] we really described only one experiment of this kind (litter 654, pp. 391 and 393), because in the second experiment only one young rat was investigated chemically and histologically from the whole litter (litter 650, p. 391). Owing to the importance of the results obtained, five new experiments were made in the present investigation.

Five pairs of adult rats were mated for this experiment. The mothers before and during pregnancy and the fathers before mating were given $N\,2$[1] diet which contained ample cod-liver oil and butter as the source of fat-soluble factor. The mothers during lactation and the young after weaning were given $-A$ diet, which was deficient in fat-soluble factor.

After birth four litters (Nos. 1–4) were each reduced to five young and one, litter No. 5, to seven young. The results obtained are shown in Tables I and III.

[1] For the composition of this and other diets mentioned in this paper see our previous paper in this journal, p. 1309.

If in these tables we compare the difference in the young born of mothers kept during pregnancy on *N* 2 diet with those born of mothers kept during pregnancy on *NB* 3, *NB* or – *A* diet, we see a definite improvement in the weight and in the degree of calcification of the skeleton of the young of the first group (mothers on *N* 2), which could only be explained by the good diet of the mother during pregnancy. These results are clear both at the day of weaning and when the young were 65 days old.

In these experiments the histological examination did not show the difference between the two groups so clearly as did the chemical analyses, because osteoporosis, or osteoporosis with slight rickets, prevailed. Histological examination was therefore not made in all litters. Slight histological changes from the normal were due to the normal and comparatively good feeding of the mother rats during pregnancy (*NB* diet). In spite of that, when cod-liver oil was added to the maternal diet during pregnancy, the chemical analyses showed quite definite improvement in the calcification of the skeleton of the young. These results prove once more the importance of chemical analyses and the unreliability of only making histological examinations in the investigation of the skeleton.

The general nourishment of the young rats (their size, state of the fur, development and condition of muscle, amount of fat in the body, presence or absence of xerophthalmia, diarrhoea, etc.) was also better in the group in which the mothers were better fed during pregnancy.

(2) *The influence of an excessive amount of calcium in the mothers' diet during pregnancy upon the growth, general nourishment and skeleton of their young.*

For this experiment two pairs of adult rats were mated. The mothers and fathers before mating and the mothers during pregnancy were fed on diet *NB* 3. This diet was similar to the normal *NB* diet (containing butter only as the fat), but the amount of calcium in it was trebled by the addition of $CaCO_3$, so that the fresh diet contained 0·77 % Ca and the air dry diet 1·18 % Ca. The mothers during lactation and the young after weaning were fed on – *A* diet, deficient in fat-soluble factor. The number of young in each litter was reduced after birth to five, ten young rats being used for the experiment.

Comparing the figures in Table II with those in Table III, it is clear that there is no difference at all between the weight or calcification of the skeleton of the *NB* 3 and the *NB* groups of rats. Their degree of nourishment, as well as the histological picture of the skeleton, also showed no essential difference.

So it can be concluded that enriching the normal *NB* diet of the mother during pregnancy with an excess of calcium does not improve the condition of the young nor, in particular, their rachitic skeleton. The marked improvement in the general and in the rachitic changes of the skeleton of the offspring kept on – *A* diet and born from mothers fed on *N* 2 diet during pregnancy, should therefore be ascribed to the beneficial influence of an excess of fat-soluble factor in the mothers' diet during pregnancy.

Table I. *Average chemical composition of the skeleton of young rats kept on a diet deficient in fat-soluble factor (− A), whose mothers before and during pregnancy and whose fathers always were fed on N 2 diet rich in fat-soluble factor and calcium. The diet of the mothers during lactation was deficient in fat-soluble factor (− A).*

	Killed when 24 days old						Killed when 65 days old					
	No. of rats taken for investigation	Av. weight of rats		In bones			No. of rats taken for investigation	Av. weight of rats		In bones		
Litter no.		♂ g.	♀ g.	H_2O %	Fresh Ca %	Dry Ca %		♂ g.	♀ g.	H_2O %	Fresh Ca %	Dry Ca %
1	2	35	32	59·6	6·1	15·2	3	68	74	46·7	7·9	15·0
2	2	40	34	57·3	6·3	14·7	3	88	88	45·2	8·7	15·8
3	2	42	40	58·5	6·3	15·1	3	120	112	41·9	11·0	18·8
4	—	—	—	—	—	—	5	85	76	46·5	9·2	17·2
5	—	—	—	—	—	—	7	82	67	45·7	9·4	17·2
Average	—	39	35	58·5	6·2	15·0	—	89	83	45·2	9·2	16·8

Table II. *Average chemical composition of the skeleton of young rats kept on a diet deficient in fat-soluble factor. Diet of the fathers (NB 3) adequate in fat-soluble factor and rich in calcium. Diet of the mothers before and during pregnancy the same as that of the fathers (NB 3); during lactation, deficient in fat-soluble factor (− A diet).*

	Killed when 24 days old						Killed when 65 days old					
	No. of rats taken for investigation	Av. weight of rats		In bones			No. of rats taken for investigation	Av. weight of rats		In bones		
Litter no.		♂ g.	♀ g.	H_2O %	Fresh Ca %	Dry Ca %		♂ g.	♀ g.	H_2O %	Fresh Ca %	Dry Ca %
6	2	34	34	64·1	4·8	13·4	3	64	52	51·9	6·8	14·2
7	2	34	33	60·3	4·8	11·9	3	42	71	52·3	6·2	13·2
Average	—	34	34	62·2	4·8	12·7	—	53	62	52·1	6·5	13·7

Table III. *The influence of different diets of mothers during pregnancy upon the young. The mothers' diet during lactation as well as that of the young after weaning was deficient in fat-soluble factor (− A)*[1].

Mothers' diet during pregnancy	Number of rats used for average	Weight of young		In bones		
		♂ g.	♀ g.	H_2O %	Fresh Ca %	Dry Ca %
	At the day of weaning (24 days old)					
N 2	10	38	34	58·4	6·2	15·0
NB 3	4	34	34	62·2	4·8	12·7
NB	63	31	30	61·4	5·0	12·7
− A	5	19	19	68·3	2·6	8·2
	When 65 days old					
N 2	25	90	85	44·5	9·3	16·6
NB 3	6	53	62	52·1	6·5	13·7
NB	62	62	53	50·9	6·8	13·8
Normal rats about 65 days old	19	140	108	43·8	11·5	20·5

[1] For this table data from previous papers have been used as well as from the present experiments.

General Conclusions.

1. When the mother's "normal" diet during pregnancy is enriched with an excess of the fat-soluble factor, there is a marked decrease in the disorders of general nutrition and in the rachitic changes in the skeleton produced in the young by a diet deficient in fat-soluble factor, even when the mother is also kept on the same insufficient diet during lactation.

2. This effect is not produced by giving an excess of calcium during pregnancy, provided that the mother's "normal" diet already contains an adequate amount of calcium.

3. Therefore, in order to decrease the above-mentioned disorders in the young, an excessive amount of fat-soluble factor and not of calcium is of great importance in the diet of the mother during pregnancy. At the same time, the importance of an adequate amount of calcium in the mother's diet is emphasised.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work, and to them our thanks are due. We wish also to express our sincere gratitude to Professor C. J. Martin for his continuous support in this investigation.

[Note, added December 1st, 1924.]

Hess and Weinstock in their recent paper [1924] came to the conclusion that the improvement of the maternal diet during pregnancy and lactation mitigated rickets to a certain degree but they were unable to obtain such striking results as we did. This probably can be explained by the fact that we used a diet deficient in fat-soluble factor only, whereas the diet used by them was also deficient in phosphorus. Therefore the improvement to be expected by these workers would certainly be less pronounced owing to the greater deficiency of the diet used.

Unfortunately Hess and Weinstock did not make any chemical analyses of the skeletons of the experimental animals and we have been able to show several times that in experiments of this kind objective chemical data are often even more important than histological, the latter being sometimes too subjective.

Recent experiments by Byfield and Daniels, M. Jones, and Goldblatt have corroborated the results obtained in our investigations. In favour of our conclusions are also results obtained by Dutcher and Kennedy, Drummond, Coward and Watson, Reigher, and Luce, who showed, that the content of fat-soluble factor in the milk and its antirachitic value depend on the diet of the cow.

Hess and Weinstock's observations on 28 women, who each took 16 ounces of cod-liver oil during pregnancy, showed somewhat similar results to those, which we obtained in our experiments on rats. The babies of 15 of these

women developed rickets, but this diagnosis could only be verified by X-ray examination in eight cases. In our experiments we also were unable to obtain protection against rickets in young rats by giving cod-liver oil to the mother during pregnancy, but were only able to decrease the severity of the rachitic disorder.

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CLXXV. A COMPARISON OF THE VALUES OF YEAST AND OF ORANGE JUICE WITH THOSE OF MARMITE AND OF DECITRATED LEMON JUICE, RESPECTIVELY, IN THE CALCIFICATION OF THE SKELETON.

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(*Received October 27th, 1924.*)

At the Lister Institute, as a source of vitamin B in diets intended to be free from fat-soluble factor, marmite or dried yeast is used, and as a source of vitamin C, orange juice or decitrated lemon juice[1].

Sometimes large differences were observed in the calcium content and rachitic changes of the skeleton of rats kept on a diet deficient in fat-soluble factor. In accordance with our previous investigations [1921, 1922, 1923], we attributed this difference almost entirely to the diet of the mother rats during pregnancy and lactation. Nevertheless, it seemed that the rats looked better and resisted accidental infections more successfully on a diet deficient in fat-soluble factor, when yeast was used instead of marmite. This might suggest the presence of a very small amount of fat-soluble factor in yeast, which amount is probably not present in marmite. This suggestion is confirmed at the Lister Institute by recent unpublished experiments of Smedley MacLean and Luce, who found a small amount of fat-soluble factor in yeast fat.

Osborne and Mendel [1920, 1922] are doubtful whether lemon juice contains more than traces, if any, of the fat-soluble vitamin. Their experiments showed the presence of vitamins A, B, and C in orange juice. They also indicated the proportions of these different vitamins present; taken volume for volume orange juice is as rich as milk in vitamin B, but somewhat less rich in vitamin A.

Taking these facts into consideration, we performed experiments to discover whether the quantity of fat-soluble factor present in the amount of yeast and orange juice used in the deficient diet could influence the deficient calcification of the skeleton of rats kept on that diet.

Two litters of rats were used for the experiment, each litter being divided into four groups: $-A$, $-AL$, $-AM$, $-ALM$. Group $-A$, consisting of four rats, received the usual diet deficient in fat-soluble factor [Korenchevsky, 1922, 2, pp. 7–9], containing yeast and orange juice.

[1] Decitrated lemon juice is prepared by the addition of $CaCO_3$ in excess to lemon juice to neutralise and precipitate citric acid. It is then filtered and the filtrate used for the diets.

Group $-AL$, consisting of four rats, had the same diet, but orange juice was replaced by the same amount of decitrated lemon juice.

The diet of group $-AM$, consisting of five rats, contained marmite instead of yeast.

The diet of group $-ALM$, consisting of five rats, contained decitrated lemon juice and marmite, instead of orange juice and yeast.

The rats were put on these special diets at the age of 26 days (litter I), and at the age of 35 days (litter II). The special feeding was continued over a period of 54 days for litter I and 56 days for litter II. When the rats were killed at the end of the experiment, it was impossible to find by gross investigation any appreciable difference in their weight and other features. Spontaneous fractures of the ribs were found in several groups. The results of the chemical investigation of the skeleton of the rats are summarised in Table I. Histological examination revealed in all four groups a picture of slight rickets with osteoporosis. This pathological condition was more accentuated in the group $-AM$ (marmite instead of yeast).

Table I. *Average chemical composition of the skeleton of the rats in each of the four groups.*

	Litter I In bones			Litter II In bones		
No. of the group of rats	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %
$-A$ (Orange juice and yeast)	52·7	7·4	15·6	47·7	8·6	16·4
$-AL$ (Decitrated lemon juice and yeast)	50·4	7·9	15·9	47·3	8·2	15·6
$-AM$ (Orange juice and marmite)	52·6	7·1	14·9	46·2	8·1	15·1
$-ALM$ (Decitrated lemon juice and marmite)	46·6	7·9	14·7	47·7	8·0	15·3
Normal rats of about the same age on normal diet	43·8	11·5	20·5	39·0	13·2	21·5

Taking chiefly into consideration the calcium content of the dry bone, we may draw the following *conclusions* from the above experiments:

1. The presence of yeast in the diet deficient in fat-soluble factor instead of a corresponding amount of marmite, only very slightly increases the calcification of the skeleton.

2. This small difference may be within the limits of the usual fluctuations in the calcification of the skeleton of rats kept on this diet. If it is possible to ascribe any significance to such a small difference, it may suggest a slightly higher content of fat-soluble factor in yeast than in marmite.

3. The presence of orange juice instead of decitrated lemon juice in an otherwise identical diet does not have any definite influence on the calcification of the skeleton.

4. For the ordinary practical purpose of preparing diets deficient in fat-soluble factor it makes no difference whether yeast is used instead of marmite, or orange juice instead of decitrated lemon juice.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work, and to them our thanks are due. We wish also to express our sincere gratitude to Professor C. J. Martin for his continuous support in this investigation.

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CLXXVI. A FURTHER NOTE ON THE VALUE OF EGG-WHITE AS THE SOLE SOURCE OF NITROGEN FOR YOUNG GROWING RATS.

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(*Received October 27th, 1924.*)

In a former communication [Boas, 1924] it was stated that a diet containing all the substances essential for normal growth, but supplying protein solely in the form of egg-white, was inadequate to support growth and health in young rats. This was contrary to the results obtained by Osborne and Mendel [1913, 1, 2; 1915], and Prof. Mendel has been kind enough to suggest that some divergence in the conditions of the experiments might account for the discrepancy. The egg-white used in my experiments was, as stated, a dried commercial sample of Chinese origin. I was not therefore justified in concluding that my observation applied to egg-white in general, but, under the circumstances, I hope Prof. Mendel will pardon my rashness.

I have since compared the value of the whites of fresh hen's eggs and this dried commercial preparation as the source of protein. In both cases the egg-white was, as in my former experiments, coagulated by heat before incorporation in the diet. The rats fed on the white from fresh English eggs grew normally and had good coats, whereas those fed upon the commercial preparation ceased to grow after they had received the diet for from four to six weeks, and the typical symptoms of loss of hair, etc., described in my former paper, appeared. These results obtained with fresh egg-white confirm the experiments of Osborne and Mendel, to whom I express my regret that I should by inference have cast any doubt upon the conclusions arrived at by them.

I am at present engaged in investigating the cause of the failure following the use of the dried egg-white, but I have not as yet been able to arrive at any definite conclusions.

My thanks are due to Prof. C. J. Martin and Dr Harriette Chick for advice and criticism, while these experiments were being carried out.

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CLXXVII. AN EXPERIMENTAL STUDY OF BROWN AND WHITE BREAD IN THE DIET OF THE RAT.

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The question of the superiority of brown over white bread has been the subject of many discussions. A number of people *assume* that brown bread has a far greater dietetic value than white bread, but there are very few experimental data to warrant such an assumption. Perhaps the point most usually quoted in favour of brown bread is its vitamin B content. It is frequently stated that white bread is lacking in this factor, and therefore brown bread *must* be the better food. That this is not so, was shown by the author in a previous communication [1924, 1]. In this paper experiments were described in which rats were kept for as long as a year on a diet of white bread, butter and salt mixture. The animals showed no symptoms indicative of lack of vitamin B, the only source of which was the white bread. From a commonsense point of view, also, it seems ridiculous to state that white bread contains no vitamin B, when yeast, used in the manufacture of white bread, is accepted as being one of the richest sources of this vitamin. From information obtained from various bakers, it is found that the amount of yeast used in bread manufacture is by no means the minute amount accepted by the general public, but varies from 2–8 % of the flour used. It is not claimed that the vitamin B content of white bread is *equal* to that of brown, but that at least *some* of the vitamin is present in white bread (sufficient to provide for the needs of the rat). Even if brown bread does contain more vitamin B than does white bread, why are some people so insistent on its use in England, a country where the ordinary mixed diet of the nation must be very rich in this vitamin? Brown bread, being made from whole-meal flour, must contain more non-digestible material (frequently designated by dietitians as "roughage") than white bread, but fruit and vegetables are even better sources of this factor. Thus from a commonsense basis there seems little reason to accept the superiority of brown over white bread. The experimental side of the question is presented in this paper.

Animals and Diets.

Two series of rats, twelve in each set, six females and six males, were fed on bread (brown or white), butter, salt mixture and water [for details of composition see Hartwell, 1924, 1]. The brown bread experiment lasted for 25 weeks and the white bread experiment for 21 weeks. The brown bread used was made from pure wheat meal, from which a portion of the endosperm,

but not the germ, had been removed[1]. It is therefore stronger in the supposedly good elements of protein and vitamin B than 100 % whole meal would be.

Growth.

On the white bread diet the females grew more rapidly than the males, the reverse of what occurs on a good mixed diet. With brown bread instead of white, the relative rate of growth was normal. At the end of 21 weeks the average weight of the males fed on brown bread was 280 g., while those fed on white bread weighed only 167 g. At this time the females on both diets were of about equal weights. The exact weights at the twenty-first week cannot be given because the females were all in different stages of pregnancy. Thus from the point of view of growth, the females grew equally well on either brown or white bread, but the males did better on the brown bread diet.

Maintenance.

The rat is regarded as fully grown at the end of a year, and as these experiments only lasted 5–6 months the effects of the two diets from the point of view of maintenance cannot be given. Since the requirements for maintenance are not greater than the demands for growth, it is probably correct to assume that both diets would meet the needs of the rat for maintenance, though possibly the males would only be getting a low ration of vitamin B on the white bread diet.

Reproduction.

There are two aspects of reproduction to consider, viz. gestation and lactation.

Gestation.

Both sets of animals produced litters, but the young were of low weight, and the litters (especially those of the mothers fed on brown bread) were small. Details are given in the following table:

White bread diet

11 litters from 6 does in 21 weeks:
- 5 litters born dead
- 0 whole litters died within one week
- 6 litters, some young of each were reared (see next table)

Brown bread diet

14 litters from 6 does in 25 weeks:
- 4 litters born dead
- 3 whole litters died within one week
- 7 litters, some young of each were reared (see next table)

White bread diet		Brown bread diet	
No. in litter	No. successfully reared	No. in litter	No. successfully reared
9 reduced to 6	4*	9 reduced to 6	6*
9 „ 6	6	8 „ 6	5*
7 „ 6	5	7 „ 6	4
6	6*	5	2*
5	3	4	—
5	4*	4	3
		4	3
		4	—
		2	2
		2	—
Average 6·85	Total 28	Average 4·9	Total 25

Control average 10 per litter

* Mother rat fed on bread and milk during lactation.

[1] I wish to thank Messrs Wheatley's Ltd. for kindly giving me this information.

Lactation.

The question of lactation needs special consideration. If the mother rat is fed on the bread diet throughout her existence, and if she then fail to rear her babes, the conclusion might be that this diet is unsatisfactory for lactation. This would not be a justifiable conclusion, because the failure to rear her offspring might be due to the unsuitability of the diet for gestation. Hence to throw light on this point, a separate series of experiments was necessary, in which the rats were fed on a good mixed diet during pregnancy, and the bread diets were only started after the birth of the litter. Again, the rat might be able to produce stronger young on a poor diet, if she had been well fed before fertilisation took place and therefore had good reserves.

The following experiments were therefore made, the results being shown in Fig. 1.

1. *Main experiment.* The rats were fed on bread (white or brown), butter, and salt mixture throughout growth, gestation and lactation. The does seldom reared all six young, even if given a diet of bread and milk from the birth of the young.

2. *Experiment A.* The rats received a good mixed diet until the young were born, after which they had the bread, butter and salt mixture diet. The mother rats were generally able to rear six young, but the growth of the sucklings was very slow. Very little difference was found in the rate of growth of the litters if the mothers received brown or white bread (butter and salt mixture in addition), but the ones belonging to the mothers fed on brown bread were, perhaps, just the better. Also, although the mothers lost weight while suckling their young, the loss was not quite so great when brown bread was used. Compared with the control animals (whose mothers had bread and milk) the rate of growth was distinctly poor. It is suggested that the slow growth of the sucklings is due to the low protein content of the diet, for the addition of small quantities of extra protein (*e.g.* 0·9 g. food casein to 15 g. bread) to the mother's diet resulted in an appreciable improvement in the growth of the young, even when butter and salt mixture were omitted from the diet [Hartwell, 1924, 2].

3. *Experiment B.* The rats were fed on a good mixed diet until old enough to breed, when they were given a white bread, butter and salt mixture diet. Four does and two bucks were used, and the females produced litters of twelve, eleven, ten, and five young respectively. They were of better weight than when the mothers had received the bread diet throughout their lives. During lactation two does were given the bread diet and two had bread and milk. Two mothers reared six young (one mother on each diet), and the other two mothers each lost one baby. These results, however, are distinctly better than when the rats had been having the bread diet since they were weaned. This is very understandable, for although the litters were all first ones (to make the experiments comparable), the mothers were heavier and better

grown animals than those fed entirely on the bread mixture. Summing up, one may say that neither brown nor white bread (with butter and salt mixture) is a satisfactory diet for the rat during gestation or lactation, and further that, in this respect brown bread has practically no advantages over white.

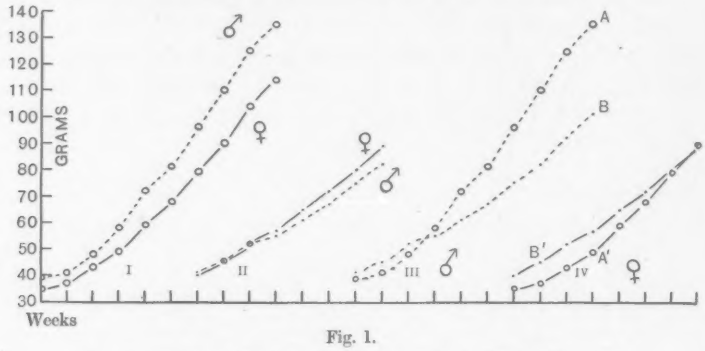

Fig. 1.

I. Brown bread diet. Comparative growth of males and females.
II. White bread diet. Comparative growth of males and females.
III. Comparative growth of males on:
A. Brown bread diet;
B. White bread diet.
IV. Comparative growth of females on:
A′. Brown bread diet;
B′. White bread diet.

McCollum, Simmonds and Parsons [1919] find that "with wheat proteins at 9 %, only an occasional litter of one or two young has been secured. None of these was weaned." In the diets used by me the protein would be approximately 10 % of the dry constituents, and the percentage of butter is also greater than that used by McCollum, etc. This may be the reason why the breeding of my animals, although below normal, was better than that of McCollum's rats.

Conclusions.

1. The rate of growth of young rats on a diet of bread (white or brown), butter and salt mixture is slow.

2. Male rats grow more rapidly on brown bread than on white. Female rats grow equally slowly on either kind of bread.

3. For gestation and lactation, brown and white bread appear to be equal. With both diets, the litters are of poor weight at birth, and only a few young are weaned.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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CLXXVIII. ESTIMATION OF URIC ACID BY BENEDICT'S METHOD.

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BENEDICT'S very useful and delicate method for the estimation of uric acid in the blood [1922, 1, 2] depends on the appearance of a blue colour in a Folin-Wu blood filtrate, on adding to it appropriate amounts of 5 % sodium cyanide solution and his special arsenic phosphotungstic acid reagent. In a blank determination, however, a blue colour also appears, which, as Benedict has pointed out, varies in intensity with different samples of sodium cyanide. To overcome this difficulty Benedict adds a trace of ammonia to the cyanide solution, whilst Folin [1922], pointing out that strongly ammoniacal solutions do not yield the maximum colour and that they show a greater tendency to produce turbidity, uses a two to three weeks old solution of 15 % sodium cyanide in 0·1 *N* sodium hydroxide. Benedict supposes that the efficacy of this latter solution is due to the spontaneous formation of ammonia in cyanide solutions. Neither of the above methods, however, completely solves the difficulty, for some colour still persists in a blank determination, with the result that, using certain samples of sodium cyanide, the colorimetric reading over a wide range is by no means proportional to the amount of uric acid present. Concerning this Benedict writes [1922, p. 192]: "Using 0·02 mg. of uric acid as standard, results are satisfactorily exact when the unknown reads between 10 and 24 mm., when the standard is set at 15 mm.... With 2 mg. of uric acid results may be about 10 % too high." And again [1922, pp. 192–3]: "...the colour from a blank is negligible inside the limits above mentioned, since it is present both in standard and unknown." It is obvious that this last statement is only absolutely true when standard and unknown have the same intensity of colour; it becomes less and less true as the disproportion between standard and unknown becomes more marked and the colour produced in a blank determination increases.

The object of the present paper is to show (1) that it is, from a purely mathematical standpoint, essential to take into account the colour given by the blank controls, and (2) that if this is done, the method gives consistently accurate results over a wide range of concentration.

Let three solutions *C* (containing 0·4 mg. of uric acid per 100 cc.), *B* (containing 0·2 mg. of uric acid per 100 cc.) and *A* (a blank determination) be of the same colour with colorimetric readings of x, y and a mm. respectively.

Then since the amount of colour-producing substance is inversely proportional to the reading (*i.e.* the depth of columns of identical colour)

$$\left(\frac{1}{x}-\frac{1}{a}\right)=2\left(\frac{1}{y}-\frac{1}{a}\right),$$

i.e.

$$y=\frac{2ax}{a+x}, \text{ or } a=\frac{xy}{2x-y}.$$

Thus if any two of the three depths x, y and a are known the third may be deduced.

Supposing when $x = 10$, $a = 35$ (as with the specimen of sodium cyanide we are now using), then

$$y = 15{\cdot}6;$$

thus solution B, containing half the amount of uric acid of solution C, reads not 20, the theoretical value if the colour in the blank is regarded as negligible (because it is present in both standard and unknown, within the limits mentioned by Benedict), but 15·6. This is shown to be true experimentally when solutions C and B are read against one another (Table I).

Table I.

	Solution C (0·4 mg. %)	Solution B (0·2 mg. %)
Theoretical (no correction for blank)	10	20
Theoretical (correction for blank)	10	15·6
Exp. 1	10	15·7
„ 2	10	15·5
„ 3	10	15·4
„ 4	10	15·5
„ 5	10	15·6

In a similar way if solution B contains an unknown quantity of uric acid, say Q mg. %, then if the standard solution C contains 0·4 mg. %,

$$\left(\frac{1}{x}-\frac{1}{a}\right)=\frac{0{\cdot}4}{Q}\left(\frac{1}{y}-\frac{1}{a}\right),$$

i.e.

$$Q=\frac{0{\cdot}4\times x(a-y)}{y(a-x)}.$$

With the specimen of sodium cyanide we are using and $x = 10$, $a = 35$,

$$\therefore\ Q=\frac{0{\cdot}4(350-10y)}{25y}=\frac{140-4y}{25y}.$$

The results shown in Table II prove that in pure uric acid solutions the intensity of colour produced is directly proportional to the amount of uric acid present when Benedict's reagents are employed and *correction is made for the colour in a blank determination.*

Taking Benedict's range of accuracy, *i.e.* readings between 10 and 24 mm. when the standard (x) reads 15 mm., and taking a as 52·5 mm., then when the unknown solution

(a) reads 24 mm. Benedict's result = 0·25 mg. %, corrected result = 0·19 mg. %, *i.e.* error approximately 30 %,

(*b*) reads 10 mm. Benedict's result = 0·6 mg. %, corrected result = 0·68 mg. %, *i.e.* error approximately 12 %.

Since none of the figures in the third column (*i.e.* corrected for blank colour) differs from the true amount present by more than ± 3 % (*i.e.* not greater than colorimeter error), the advantage of the correction is evident.

Table II.

	Uric acid in mg. % in pure solution	Amount given by direct relation, *i.e.* $\frac{0\,4x}{y}$	Amount given when corrected for blank colour
1	0·800	0·699	0·816
2	0·600	0·537	0·592
3	0·533	0·485	0·519
4	0·300	0·327	0·297
5	0·266	0·306	0·269
6	0·200	0·258	0·201
7	0·133	0·211	0·135

The above results are specimens of many repeated experiments.

It should be added that we have been unable either by Benedict's or Folin's method of reducing the colour in the blank determination to reduce the value of "*a*" below 60 ($x = 10$), with the specimens of sodium cyanide used. It is therefore necessary to determine the value of "*a*"[1] for the particular reagents employed, and correct the final results accordingly.

It will be seen from the above discussion and Table II that the uncorrected results are too high when the amount of uric acid present is less than 0·4 mg. % (*i.e.* the amount of uric acid in the standard solution), and too low when the amount of uric acid in the unknown solution is greater than that in the standard. The correction probably serves, at any rate in part, to explain why the Benedict method gave apparently higher results than the Folin-Wu method, since as Benedict himself writes [1922, 1, p. 203], "the difference between the two methods tends to disappear or is reversed as soon as the uric acid by the Folin-Wu method approaches a figure of 3·5 to 4·0 mg."

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[1] This can be done in less than 10 minutes, and may be repeated each week.

CLXXIX. THE POLYMERISATION OF FORMALDEHYDE IN PRESENCE OF INORGANIC SUBSTANCES.

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(*Received October 31st, 1924.*)

Although most of the experimental evidence is in favour of Baeyer's suggestion [1870] that the first stage in the natural synthesis of sugars is the production of formaldehyde from carbon dioxide and water, and that the sugars are produced by the polymerisation of this aldehyde, the correctness of both stages has been questioned by some investigators. One of the difficulties met with is the absence of proof that formaldehyde exists in growing leaves as a definite intermediate stage.

Moore and Webster [1914, 1918] proved that formaldehyde is formed by the action of ultraviolet light on carbon dioxide and water in presence of certain inorganic substances, such as ferric chloride, and also that formaldehyde is polymerised to reducing sugars in ultraviolet light. Nevertheless these workers consider that formaldehyde is not an intermediate in the photosynthesis of carbohydrates since if it were formed it would combine with the proteins rather than polymerise to sugars.

Baly, Heilbron and Baker [1921] and Baly [1922] have found that the function of the inorganic materials used by Moore and Webster is to stabilise the photochemically active formaldehyde and so prevent it from polymerising to sugars. They also showed that if malachite green or *p*-nitrosodimethylaniline be present, formaldehyde is formed from carbon dioxide and water under the action of visible light. They claim that in the leaf the activated formaldehyde primarily formed immediately polymerises to sugars with the result that the formation of formaldehyde in sufficient quantities for its ready recognition in the growing leaf is not to be expected.

By the action of ultraviolet light on formaldehyde solutions, Baly [1922] obtained several hundred grams of concentrated sugar syrup consisting of hexoses only.

Ewart [1919, 1920] repeated and extended the experiments of Butlerow, Loew and Tollens on the polymerisation of formaldehyde in presence of alkalies and concluded that formaldehyde does not form a stage in the synthesis of sugars in plants. The main arguments brought forward are: (1) that every

method of polymerising formaldehyde to sugars yields a mixture of sugars in which pentoses are included, and often form the main yield, whereas pentoses are not direct products of photosynthesis in plants; (2) alkalies do not polymerise formaldehyde in the cold, unless to a very slight extent, but produce instead methyl alcohol and formates. Pronounced sugar formation takes place only when the alkali acts on a polymer or hydrate of formaldehyde, and even then the process is a wasteful, indirect way of producing sugars.

Baly's production of hexoses, unmixed with pentoses or trioses, by the illumination of formaldehyde solution in ultraviolet light disposes of the first objection.

The second objection will be examined in some detail. Ewart found that at room temperature only traces of reducing sugars were produced when formaldehyde solutions are exposed to the action of sodium hydroxide and calcium chloride for three months. At the boiling point larger yields were obtained. He found that in general much more sugar was produced in the presence of hydroxides of bivalent metals than in that of equivalent amounts of alkali hydroxides.

Since results of experiments carried out at the boiling point are of doubtful application to the elucidation of the mechanism of sugar formation in nature, we have investigated the influence of various inorganic substances upon weak solutions of formaldehyde at room temperatures. The investigation was undertaken in consequence of an observation made in the Biological Department of this College by Mr. T. L. Lancaster, who found that sugars were produced in considerable quantity on the outside of a type of specimen jar containing various botanical specimens, preserved in 5 to 10 % solutions of commercial formalin. Sugar was produced only on those jars which were exposed to sunlight and were closed by a metal cap.

Analysis of the metal covers showed that they consisted of zinc containing about 0·24 % of magnesium but free from arsenic and cadmium. That the formation of sugar was not due to some material contained in the botanical specimens was shown by the fact that a specimen of 5 % formalin gave rise to sugars when exposed to sunlight in these containers. The metal covers were considerably corroded and the white powdery incrustation was found to consist of basic carbonates mixed with a small quantity of formates.

A solution of formaldehyde in water was obtained by heating paraform in a current of nitrogen and passing the gas so obtained into distilled water. The strong solution so obtained was diluted to a strength approximately equivalent to 5 % formalin and the concentration accurately determined by the potassium cyanide method of Elvove [1911], which was found to be the most accurate when the solutions are dilute and least influenced by the presence of sugars. The "catalysts" used were all carefully purified. In each case 2 g. of the "catalyst" was employed and was immersed in the liquid or suspended above it in a small specimen tube. 50 cc. of formaldehyde solution were used in each case and the 180 cc. flask which contained the reactants was sealed

by a cork and paraffin-wax. The flasks were placed in a position where they were exposed to bright sunlight for as long a period each day as possible, and were left for 18 months. The following table shows the qualitative results obtained:

Catalyst	Catalyst in vapour. Loss of formaldehyde %	Catalyst in liquid. Loss of formaldehyde %
Metal cover	34·3	4·2
Magnesium	13·2	17·4
Zinc	21·2	20·8
Basic zinc carbonate	3·8	3·6
Zinc oxide	1·4	24·2
Magnesium oxide	3·8	7·2
Magnesite	0·6	2·8
Zinc oxide and magnesium oxide	6·2	32·2
Magnesite and basic zinc carbonate	13·3	26·4

After the above determinations had been made there remained about 20 cc. of solution in each case. These residues were united and the content of methyl alcohol was determined by Bamberger's method [1904]. The solution was found to contain less than 0·1 g. of methyl alcohol. Thus the total amount of methyl alcohol in the illuminated solutions would be less than 0·25 g., and thus, of the total amount of formaldehyde which had disappeared, less than 22 % could have been converted into methyl alcohol and formic acid.

A current of air was passed through the remainder of the solution, water being added from time to time, until the smell of formaldehyde disappeared. The solution was then concentrated *in vacuo* and traces of inorganic salts were precipitated by the addition of a small quantity of alcohol, and were filtered off. The filtrate was further concentrated *in vacuo* and yielded 0·4 g. of a thick yellow syrup. This syrup contained reducing sugars; it was partially fermentable and appeared to contain both pentoses and hexoses. The appearance of the osazone showed that a number of sugars were present, but phenylglucosazone appeared to predominate.

Conclusion.

The results appear to show that various inorganic substances have a considerable catalytic influence on the polymerisation of weak solutions of formaldehyde to sugars in the presence of sunlight. In this case the sugars are not a by-product of the reaction in which methyl alcohol and formic acid are produced. The reaction appears to be slow under the conditions described, but we hope to show that the rate of reaction can be accelerated considerably by a suitable modification of the conditions. Since the results now communicated are of a preliminary nature we propose to postpone a detailed consideration of their bearing upon the photosynthesis problem until the results of experiments now in progress are available.

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CLXXX. THE EFFECT OF IRRADIATION OF THE ENVIRONMENT WITH ULTRA-VIOLET LIGHT UPON THE GROWTH AND CALCIFICATION OF RATS, FED ON A DIET DEFICIENT IN FAT-SOLUBLE VITAMINS. THE PART PLAYED BY IRRADIATED SAWDUST.

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(*Received October 31st, 1924.*)

Introductory.

In previous publications [Hume, 1922; Hume and Smith, 1923] evidence was brought forward to show that growth can be induced temporarily in young rats, fed on a diet deficient in fat-soluble vitamins, by exposing them to a source of ultra-violet radiation, and that the same effect is produced indirectly, when the rats are not themselves irradiated, but when their immediate environment only has been exposed to ultra-violet light. It was concluded that this latter result was due to some property conferred upon the air by the ultra-violet rays. The first of these observations was made simultaneously by Goldblatt and Soames [1922] and confirmed by Steenbock and Nelson [1923], but Webster and Hill [1924] have failed to confirm the second.

In the previous experiments, glass jars of 8 litres capacity were exposed daily for 10 minutes to the radiations and the experimental rats subsequently allowed to inhabit them. Sawdust, used for bedding, was present in the jars during irradiation and afterwards, but the possibility of its complicity was completely neglected. In the experiment of Webster and Hill, sawdust was not present during irradiation. The experiments have therefore been repeated to determine whether the presence of sawdust is the cause of the discrepancy, and it may be stated at once that the result of irradiating jars without sawdust has proved negative. In the presence of sawdust, however, the effect of irradiating the jars was not significantly inferior to that obtained by irradiating the animals directly.

Technique.

The experiments were carried out with two different diets and with two different mercury-vapour quartz lamps.

Both diets were deficient in fat-soluble vitamins and differed only in the source of protein. In the first series the protein was Chinese egg-white and 10 % caseinogen, and the diet had the following composition:

Chinese egg-white (dried)	360 g.	Marmite	100 g.
Caseinogen	40	Lemon juice	100
Starch	1000	Hardened cotton seed oil	300
Salt mixture	100	Distilled water	1800

The composition of the salt mixture was as follows:

Sodium chloride ...	51·9 g.	Calcium phosphate ...	162·0 g.
Magnesium sulphate ...	164·0	Calcium lactate... ...	390·0
Sodium acid phosphate	104·1	Ferric citrate	35·4
Potassium phosphate ...	286·2		

The growth and general condition of the rats on the diet containing egg-white[1] were not as good as had been previously observed in similar experiments when caseinogen was used as the sole source of protein. A second series of experiments was therefore set up, which only differed from the first by the substitution of an equal weight of caseinogen for the egg-white previously used. All caseinogen used had been heated in an electric oven at 120° for two days and one night and was stirred every hour throughout the day time.

The mercury-vapour quartz lamps used were both made by the Hewittic Electric Co. One was a *Y* 4 Pistolette lamp (to be referred to as the "old lamp"), which had been in use for some considerable time; on a 200 volt circuit it was using a current of 3·5 ampères.

The second lamp (to be referred to as the "new lamp") had a straight burner with an arc about 10 cm. in length; on a 200 volt circuit it was using a current of 4 ampères. It was perfectly new at the beginning of the experiment.

A photograph of the spectra of both lamps was taken by Messrs Adam Hilger with one of their small quartz spectrographs (photographic range 8000 Å.–1850 Å.) on Hilger Schumann plates. Their report states: "The old lamp radiates wave lengths down to 1900 Å. very feebly. The new lamp radiates as far as 1860 Å. with considerable intensity, though the strong mercury line at 1849 Å. could not be recorded." There was thus a very marked difference between the two lamps and if the plates are compared it is seen that, in order to produce the same effect with the shorter wave lengths, it was necessary to expose the plate for about twelve times as long to the old lamp as to the new.

In the first series of experiments, in which the basal diet contained both egg-white and caseinogen, both lamps were used for parallel groups of experimental animals, in order that a comparison between the lamps might be made. In the second series, in which the protein was caseinogen alone, the new lamp only was used.

[1] This commercial egg-white was subsequently found to be unsuitable and not equivalent in nutritive property to the white of fresh eggs [see Boas, 1924, 2].

The rats used were all black and white; they were bred on a not too rich basal breeding diet, consisting of milk, brown or white bread, carrot or cabbage, and scraps of lean meat. They were required to weigh 50 g. at 4½ weeks of age, in order to be considered normal and suitable for this type of experiment. At that weight and age they started the experiment, and litters and sexes were distributed as equally as nature would permit between the different groups of an experiment, in order that the groups might all be comparable with one another and the unnecessary multiplication of controls be avoided. The latter precaution was particularly important, since every experiment required two groups of controls, one to show the performance of the animals on the deficient diet when no treatment was applied, and one to show the maximum performance possible, when direct irradiation with ultra-violet light was employed.

The experimental animals were kept in a room facing east and the blind was kept down in summer in the early hours of the morning, when there was direct sunlight. Every rat was kept separately in a cylindrical 8 litre glass jar, with sawdust at the bottom, except where it is specially stated that a round deal board, fitting the bottom of the jar, was used instead, as a part of the experiment.

Whenever the glass jars were irradiated, as previously described [Hume and Smith, 1923], the rat was lifted out and the jar was taken to the lamp in another room where it was left directly under the lamp for 10 minutes, with its base at about 70 cm. distance. It was then covered and carried back to the animal room, the animal was placed in the jar and the cover was replaced and left on for 10 minutes. The cover was then removed, the rat remained in the jar, and if the experiment was one in which the jar was irradiated without sawdust, the sawdust and food, which had previously been removed with the rat, were replaced. When the jar was irradiated with sawdust, small particles of food were also sometimes present during irradiation, but it is known from other unpublished experiments that this is not the determining factor. The sawdust used was the ordinary mixed sawdust, mainly deal, from a sawmill; no precaution has so far been taken to use only sawdust from one kind of wood.

In the experiment where deal boards were substituted for sawdust, the jar with its wooden disc was irradiated exactly as if it contained sawdust, or else the board alone was carried to the lamp and irradiated for 10 minutes, at about 70 cm. distance; it was then brought back and placed in the jar, the rat was put in also and the cover was put on for 10 minutes, after which the rat continued to live in the jar on the piece of board, exactly as in the case of sawdust.

Animals irradiated directly were exposed in an open wire cage for 10 minutes, at 70–80 cm. distance, to the old lamp, and later also in the same way for 5 minutes to the new lamp. At first, however, when the new lamp was perfectly new, it was necessary to shield the rats' eyes even during a

5 minutes' exposure, or the animals developed conjunctivitis and failed to thrive.

All irradiations took place every day except on Sunday. The experiments lasted for 60–90 days, by which time the growth response, even to direct irradiation, was ceasing.

Calcium estimations on the bones of all animals were made by the method of Aron and Sebauer [1910]. The femora, tibiae and fibulae of each animal were dissected out and the water content and proportion of calcium were determined.

Details of Experiments.

Series I. Basal diet containing egg-white and 10 % *caseinogen.*

(A) *Irradiation with old lamp.* Chart I shows the growth of four groups of rats in which the irradiation was carried out with the old lamp.

Group 1 is a group of five animals which inhabited glass jars which were exposed for 10 minutes daily, with sawdust in the bottom, to the mercury-vapour lamp.

Group 2 is a control group of two animals, exposed directly to the radiations of the mercury-vapour lamp, for 10 minutes daily, in an open wire cage.

Group 3 is a group of five animals inhabiting similar jars, but in which the sawdust was not present during irradiation of the jar.

Group 4 is a control group of four animals, fed the basal diet, and receiving no additional treatment.

None of the animals shows really good normal growth, not even those exposed directly to the radiations of the lamp, but the growth in that group and in the group where the jars were irradiated with sawdust in them, is very definitely superior to that in the group of untreated controls. The growth of the group in which the jars were irradiated without sawdust, is indistinguishable from the growth in the group of untreated controls.

Early symptoms of xerophthalmia were apparent in most cases but appeared to be equally distributed throughout all groups. The general condition of the animals, towards the close of the experiment, which had lasted about two months, was very poor; their coats were often bad, with bald patches, particularly in the group directly irradiated, while some animals in all groups had sore, swollen and oedematous paws, ears or lips. The general condition of the animals in Series I B, also on the egg-white basal diet, was entirely similar and the condition is attributed in large part to that diet [cf. Boas, 1924, 1]. The symptoms described were not observed in Series II, in which Chinese egg-white was not used as source of protein.

(B) *Irradiation with new lamp.* Chart II shows the growth of four groups of rats in which the irradiation was carried out with the new lamp.

Group 1 is a group of five animals, inhabiting jars irradiated with sawdust for 10 minutes daily.

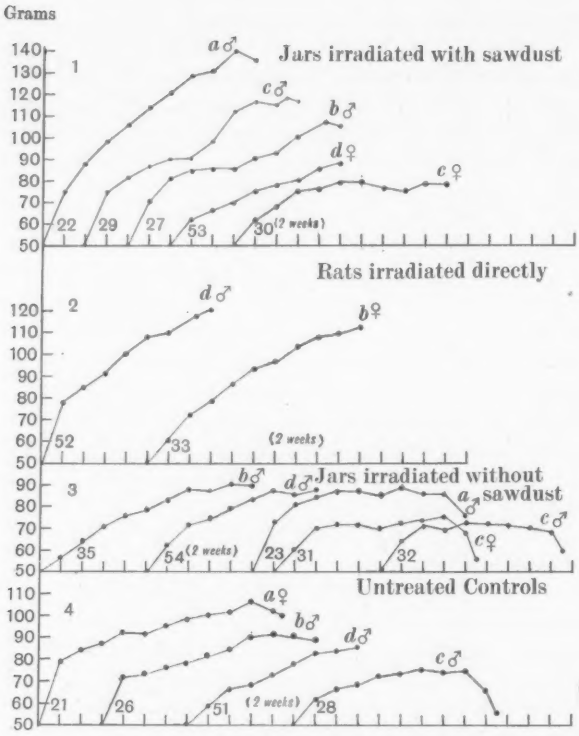

Chart I. Growth of rats in Series I A, fed on a diet deficient in fat-soluble vitamins, in which the protein was Chinese egg-white and 10 % caseinogen. Irradiation with the old lamp.

(1) Rats inhabiting jars irradiated with sawdust in them.
(2) Rats irradiated directly.
(3) Rats inhabiting jars irradiated without sawdust in them.
(4) Untreated controls.

Members of the same litter are indicated by the same small letter.

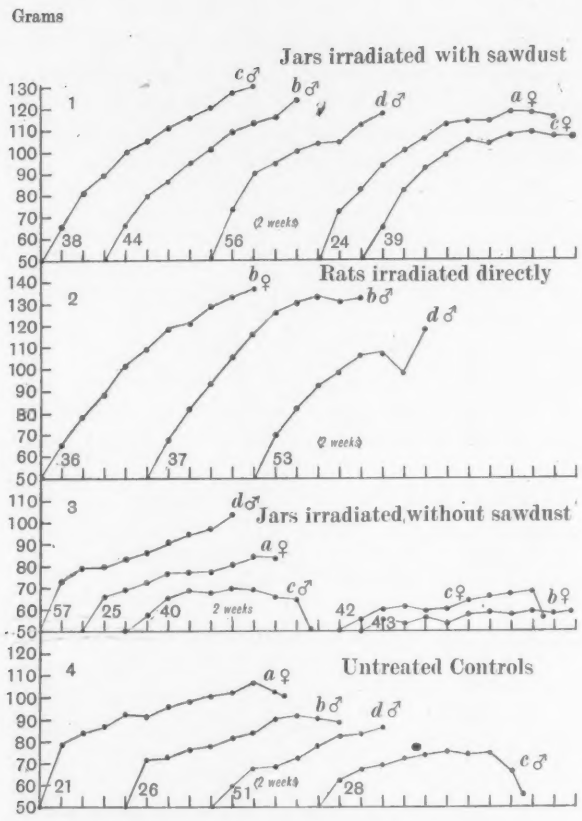

Chart II. Growth of rats in Series I B, fed on a diet deficient in fat soluble vitamins, in which the protein was Chinese egg-white and 10 % caseinogen. Irradiation with the new lamp.

(1) Rats inhabiting jars irradiated with sawdust in them.
(2) Rats irradiated directly.
(3) Rats inhabiting jars irradiated without sawdust.
(4) Untreated controls.

Members of the same litter are indicated by the same small letter.

Group 2 is a control group of three animals, exposed for five minutes daily to the direct rays of the lamp, with the eyes shielded.

Group 3 comprises five animals, inhabiting jars irradiated without sawdust for 10 minutes daily.

Group 4 is the same group of untreated controls as is shown in Chart I.

The result obtained in these four groups, in which the new lamp was used, corresponds with that obtained when the old lamp was used, save that, in the two groups where a growth response took place, that response is distinctly better than in the corresponding groups treated with the old lamp. This result is no doubt due to the greater potency of the new lamp; an exposure

Table I. *Data of the growth and calcification of the bones of the rats in Series I A and I B, fed on the basal diet containing egg-white and* 10 % *caseinogen as source of protein, and receiving various kinds of irradiation with mercury-vapour quartz lamp.*

Experimental group	Rat no.	Litter no.	Sex	Maximum weight (g.)	% water in bone	% Ca in dry bone	Duration (days)
Untreated controls	26	*b*	♂	93	41·0	15·5	74
	28	*c*	♂	75	44·8	13·5	67
	21	*a*	♀	107	39·5	Lost	74
	51	*d*	♂	88	47·0	16·6	62
			Av.	91	43·1	15·2	
Jars irradiated without sawdust. "Old lamp"	35	*b*	♂	90	42·6	15·8	72
	31	*c*	♀	75	47·5	16·0	67
	32	*c*	♂	73	45·6	14·1	60
	23	*a*	♂	88	39·4	15·4	70
	54	*d*	♂	88	44·3	15·6	62
			Av.	83	43·8	15·4	
Jars irradiated without sawdust. "New lamp"	43	*b*	♀	61	41·4	14·2	70
	40	*c*	♂	71	50·7	14·6	60
	42	*c*	♀	71	47·0	15·4	66
	25	*a*	♀	85	38·9	17·6	75
	57	*d*	♂	103	43·5	15·8	61
			Av.	78	44·3	15·5	
Jars irradiated with sawdust. "Old lamp"	27	*b*	♂	108	43·5	16·5	73
	29	*c*	♂	119	40·2	18·2	74
	30	*c*	♀	79	43·9	16·6	74
	22	*a*	♂	140	38·4	18·6	74
	53	*d*	♀	190	45·3	19·3	63
			Av.	107	42·2	17·8	
Jars irradiated with sawdust. "New lamp"	44	*b*	♂	124	38·6	18·3	70
	38	*c*	♂	137	40·6	19·3	74
	39	*c*	♀	109	42·3	19·9	74
	24	*a*	♀	118	32·3	21·8	74
	56	*d*	♂	117	39·9	17·7	61
			Av.	121	38·7	19·4	
Rats irradiated directly. "Old lamp"	33	*b*	♀	113	36·6	20·1	73
	52	*d*	♂	123	37·8	19·7	62
			Av.	118	37·2	19·9	
Rats irradiated directly. "New lamp"	36	*b*	♀	138	35·7	21·7	75
	37	*b*	♂	134	33·8	19·7	73
	53	*d*	♂	124	45·3	19·2	62
			Av.	132	38·3	20·2	

of only 10 minutes might well be an insufficient time to enable the old lamp to evoke the maximum growth response possible from the rats.

The general condition of the rats and the incidence of xerophthalmia were as has already been described for Series I A.

The values for the calcium estimations on the bones of each individual rat in Series I A and I B are set out in Table I, together with the value for the maximum weight attained. The values for calcification are seen to follow the values for growth very closely, though the unequal distribution of the sexes, as between the different groups, disturbs the comparison somewhat. Females show, as a rule, better calcification for less degrees of growth than do males. In experiments of this type, *i.e.* carried out within the first 60 days after the rat has been placed upon the deficient diet, it would appear that the response by growth and by calcification to the methods of irradiation applied are very closely parallel, when the rats have been bred as described.

Series II. Basal diet containing caseinogen as sole source of protein.

Irradiation with the new lamp only. Chart III shows the growth of six groups of rats, in which the irradiation was carried out with the new lamp.

Group 1: five animals inhabiting jars, irradiated with sawdust for 10 minutes daily.

Group 2: six untreated controls.

Group 3: five control animals exposed directly to the radiations of the lamp for 5 minutes daily in an open wire cage.

Group 4: five animals inhabiting jars, irradiated without sawdust for 10 minutes daily.

Group 5: three animals inhabiting jars, irradiated for 10 minutes daily, with a circular piece of deal board instead of sawdust in the bottom.

Group 6: three animals inhabiting jars into which a piece of deal board, irradiated for 10 minutes daily, was placed, upon which the rats lived.

The animals irradiated directly or inhabiting jars irradiated with sawdust in them, show good normal growth for about 10 weeks; those irradiated directly are if anything ultimately slightly inferior to those whose jars were irradiated while containing sawdust.

The animals inhabiting jars irradiated without sawdust differ little from the untreated controls. In Table III, the maximum weights of the animals in all the groups are set out and the individuals are so arranged that those of the same sex and litter can be directly compared.

The last two groups in Series II, in which wooden boards were used instead of sawdust, are not perfectly comparable, either in the distribution of the sexes or in the litters used, with the first four groups of that series. Both groups contain a preponderance of females and they appear to show a growth response intermediate between the maximum and minimum effects obtained in the other groups, if the growth is compared with that of the corresponding female controls. It is pretty clear that there is some effect, but it appears to be the

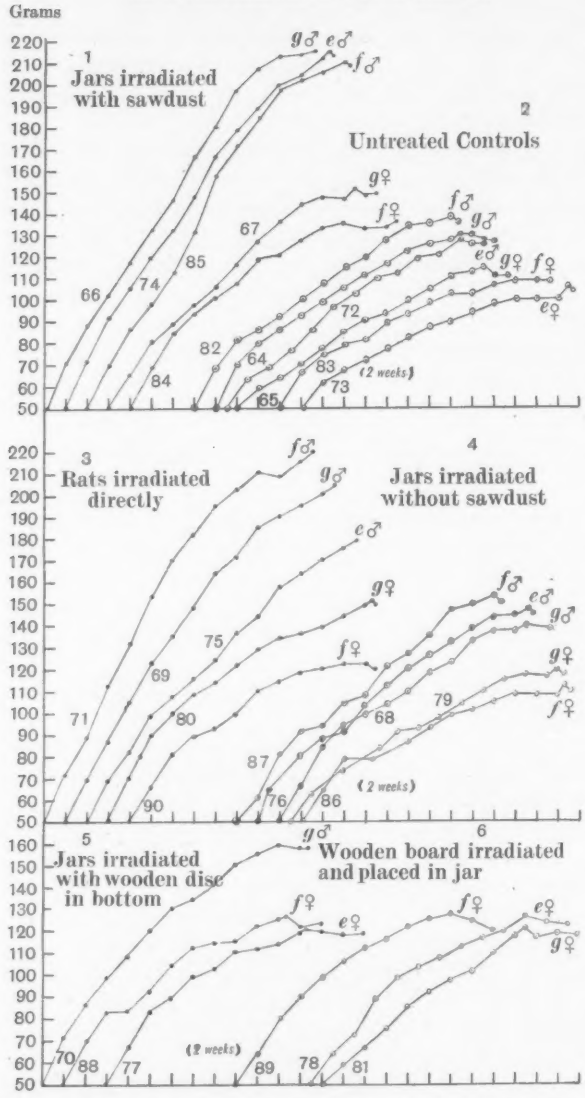

Chart III. Growth of rats in Series II, fed on a diet deficient in fat-soluble vitamins, in which the protein of the diet was caseinogen only. Irradiation with the new lamp.

(1) Rats inhabiting jars irradiated with sawdust in them.
(2) Untreated controls.
(3) Rats irradiated directly.
(4) Rats inhabiting jars irradiated without sawdust in them.
(5) Rats inhabiting jars irradiated with discs of deal board in the bottom of them.
(6) Rats inhabiting jars in which pieces of deal board irradiated separately were placed.

Members of the same litter are indicated by the same small letter.

Table II. *Data of the growth and calcification of the bones of the rats in Series II, fed on the basal diet, containing caseinogen as source of protein, and receiving various kinds of irradiation with mercury-vapour quartz lamp.*

Experimental group	Rat no.	Litter no.	Sex	Maximum weight (g.)	% H_2O in bone	% Ca in dry bone	Duration (days)
Untreated controls	72	*e*	♂	129	40·5	19·0	89
	73	*e*	♀	105	39·0	18·3	89
	82	*f*	♂	138	38·9	19·0	87
	83	*f*	♀	108	36·5	18·8	88
	64	*g*	♂	130	39·1	17·3	89
	65	*g*	♀	114	39·0	18·4	90
				Av. 121	38·8	18·4	
Jars irradiated without sawdust	76	*e*	♂	147	39·2	19·9	88
	87	*f*	♂	153	39·5	19·8	87
	86	*f*	♀	112	42·4	19·2	88
	68	*g*	♂	139	39·3	18·6	89
	79	*g*	♀	120	36·0	20·3	88
				Av. 134	39·3	19·5	
Jars irradiated with wooden board	77	*e*	♀	122	34·7	20·8	84
	88	*f*	♀	126	37·9	21·0	84
	70	*g*	♂	159	35·3	20·3	86
				Av. 136	35·9	20·7	
Wooden board irradiated alone and placed in jar	78	*e*	♀	127	34·7	21·5	85
	89	*f*	♀	127	36·1	21·5	84
	81	*g*	♀	121	36·6	20·4	85
				Av. 125	35·8	21·1	
Jar irradiated with sawdust	74	*e*	♂	215	35·2	21·1	88
	85	*f*	♂	210	33·2	21·6	86
	84	*f*	♀	137	31·9	21·9	86
	66	*g*	♂	215	33·7	22·1	88
	67	*g*	♀	151	30·0	22·9	88
				Av. 186	32·8	21·9	
Rats irradiated directly	75	*e*	♂	179	36·3	22·5	88
	71	*f*	♂	220	32·8	22·8	89
	90	*f*	♀	124	36·3	22·7	86
	69	*g*	♂	205	33·4	22·8	88
	80	*g*	♀	151	35·2	22·7	87
				Av. 176	34·8	22·7	

Table III. *Maximum growth in g. of the animals in Series II, arranged according to litter.*

Litter no.	Sex	Untreated Control	Jar irradiated *without* sawdust	Jar irradiated with wooden board	Wooden board irradiated alone	Jar irradiated *with* sawdust	Rat irradiated directly
e	♂	129	147	—	—	215	179
	♀	105	—	122	127	—	—
f	♂	138	153	—	—	210	220
	♀	108	112	126	127	137	124
g	♂	130	139	159	—	215	205
	♀	114	120	—	121	151	151

same, whether the board and jar are irradiated together or whether the board is irradiated separately.

It should be noted that the rats from time to time gnawed the boards or consumed some of the sawdust in those experiments in which sawdust was used. It is possible at times to identify quite considerable quantities of sawdust, both macroscopically and microscopically in the faeces of rats bedded on sawdust. Even when the animals do not actually pick the particles of wood up and eat them intentionally, it is not possible for them to avoid ingesting a certain amount, sticking to their food.

Symptoms of xerophthalmia, often only very slight, were apparent in most animals and occurred indiscriminately in all the groups. The general condition of the animals was much better in Series II than in Series I A and I B, in which the protein of the diet was principally Chinese egg-white. This may be explained by the unfortunate use of a particular sample of dried egg-white (see note to p. 1335).

The values for the calcium estimations on the bones of each individual rat, and for the maximum weight attained by each animal, are set out in Table II. As in Series I A and I B, the values for calcification follow those for growth very closely, but in the present series both sets of values are higher. In the first series the average maximum values for growth, throughout all the groups, ranged from 78 to 132 g., and for calcification from 15·4 to 20·2 % Ca in the dry bone. In the second series the corresponding values were 121 to 186 g. and 18·4 to 22·7 % Ca. The second series was carried on slightly longer than the first, but not sufficiently so to affect the calcification significantly, and both series were continued until it was deemed that the full growth response had been obtained.

The calcification of the bones of the animals irradiated directly in Series II is normal or even super-normal. Korenchevsky [1922, p. 65] gives the normal average calcification at the age of from 80 to 120 days as 21·5 % Ca in the dry bone. The animals which inhabited jars irradiated with sawdust in them give an average value of 21·9 %, which must therefore be regarded as normal also, though it is slightly inferior to that given by the animals irradiated directly, *i.e.* 22·7 %.

Discussion.

The results so far obtained throw no light upon the question of how the presence of sawdust in the jars during irradiation influences the growth of rats subsequently in contact with it. While the present paper was in preparation, a communication by Steenbock and Black [1924] was published, showing that an effect upon growth and calcification, similar to that here described, can be obtained by irradiating the food upon which the rats are fed. These workers advance no theory as to the mechanism but it would seem probable that the underlying phenomena are the same in both cases. In the present series of experiments, the rats undoubtedly consumed some of the sawdust,

but experiments now in progress suggest that actual ingestion of the irradiated sawdust is not necessary.

The recent observations of Kugelmass and McQuarrie [1924], that substances curative of rickets are capable of fogging a photographic plate through a quartz screen, are also interesting in this connection.

Summary.

1. The interpretation of a previous series of experiments, in which the increased growth obtained with rats fed on a diet deficient in fat-soluble vitamins, and inhabiting jars previously irradiated, was ascribed to some property impressed upon the air contained therein, has now been found to be unjustified.

2. It has now been found that when sawdust was present in the jars during irradiation, the rats subsequently inhabiting them showed growth as good as that shown by directly irradiated animals.

3. When sawdust was not present during irradiation of the jars, the animals showed growth similar to that of untreated controls.

4. When a piece of deal board was irradiated instead of sawdust, either in the glass jar or separately, the rats gave an intermediate growth response.

5. Estimation of the calcium content of the bones showed that the degree of calcification, when sex was duly taken into account, was directly proportional to the amount of growth.

6. No evidence has so far been obtained as to how the sawdust cooperates. Further experiments are in progress to investigate the problem.

The thanks of the writers are in particular due to Professor C. J. Martin for much wise advice, to Dr Harriette Chick for very kindly supervising the calcium estimations in Series I, and to the Medical Research Council for the remuneration of the workers.

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ADDENDUM.

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NOTE UPON THE EFFECT ON THE GROWTH OF RATS, RECEIVING A DIET DEFICIENT IN FAT-SOLUBLE VITAMINS, OF EXPOSING THEIR ENVIRONMENT TO THE EMANATION FROM RADIUM BROMIDE.

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The following observations were made shortly after the publication of an earlier paper by Hume and Smith [1923]. The object was to examine the suggestion made by these authors that the stimulating effect upon the growth of rats upon a $-A$ diet, observed when their environment (glass jars containing sawdust litter) was irradiated with ultra-violet light, might be due to increased ionisation of the air contained in the jar. Increased ionisation is an effect of exposing air and other substances to ultra-violet radiations. X-rays and the γ rays from radium compounds are, however, much more powerful ionising agents than ultra-violet light, and the following experiments, in which rats on a $-A$ diet occupied an air space exposed daily to the emanation from radium bromide, were made to determine whether ionisation of the air was concerned in the effect observed by Hume and Smith. Since our experiments were carried out, these authors, on the basis of the later experimental work contained in the above paper, have altered the opinion expressed in their first paper and have reached the conclusion that the beneficial effect upon growth was due to the irradiation of the sawdust present in the glass jars. The negative results detailed in this note indicate that a temporary ionisation of the air has no effect in promoting growth, and played no part therefore in producing the effect observed by Hume and Smith.

The experiments were carried out as follows.

The rats inhabited glass jars of 8 litres capacity, containing sawdust at the bottom for bedding and closed at the top with gauze metal lids. The rat, sawdust and any scraps of food were removed from the jar and the empty vessel carried to another room where radium was suspended in the jar for ten minutes, the jar being covered with a glass lid. The radium used was 10 mg. of radium bromide, enclosed in a glass tube. The radium being removed, the glass cover was replaced and the jar carried back immediately to the rat room. The rat was at once placed in the covered jar and allowed to remain for ten minutes; the glass cover was then taken away, the sawdust replaced and the rat continued to live in the jar. This procedure was carried out on six days in the week and the observations lasted for five to seven weeks.

The rats whose homes were thus treated were compared with control animals from the same litters, also receiving $-A$ diet, but living in glass jars subjected to no form of radiation.

The effect upon the growth of the rats of treating the jars with the radium emanation was negative in every case.

Three experiments were made with three separate litters of young rats, during the months of April and May. The three litters were somewhat differently bred and the young animals of the respective litters presumably possessed differing reserves of fat-soluble vitamins, for they reacted differently, as regards their growth, when placed upon the deficient diet. The observations were begun with animals of about 50 g. weight in each case. The diet was the $-A$ diet usually employed in this Institute, with the protein given in the form of casein, and was the same as that used by Hume and Smith in series II of the foregoing paper.

Exp. I. The control male rats of litter 1 (seven rats) grew at almost the normal rate during the seven weeks of the experiment (see Table I); those living in jars exposed daily to the radium emanation grew no better. In the case of the females there was also no difference between the two groups of animals.

Exp. II. In Exp. II (litter 2, three rats), the growth of the control animal showed an immediate check when placed upon the $-A$ diet; the growth of the animals in the exposed jars was similar (see Table I).

Exp. III. In Exp. III (litter 3, eight rats), there was also no significant difference in the growth shown by the two different groups of rats. It is always necessary, of course, to compare males with males and females with females.

Table I. *The effect of exposing the air in jars, inhabited by young rats on $-A$ diet, to the emanation from radium bromide. Time of exposure, 10 minutes daily.*

Rat no.	Sex	Time of expt. (days)	Body weight, g. Initial	Final	Difference	
Exp. I. Litter 1						
1	♀	44	51	113	62	Exposure of jar to radium after 7th day
2	♀	44	49	110	61	" " " " "
3	♀	44	59	123	64	Control, no exposure
4	♂	48	53	146	93	Exposure of jar to radium after 7th day
5	♂	48	54	135	81	" " " " "
6	♂	48	57	120	63	Control, no exposure
7	♂	48	56	173	117	" "
Exp. II. Litter 2						
8	♀	38	49	83	34	Exposure of jar to radium
9	♀	38	50	81	31	" " "
10	♀	38	51	87	36	Control, no exposure
Exp. III. Litter 3						
13	♀	35	51	91	40	Exposure of jar to radium
14	♀	35	49	92	43	" " "
17	♀	35	48	88	40	Control, no exposure
18	♀	32	50	89	39	" "
12	♂	35	51	103	52	Exposure of jar to radium
19	♂	29	49	86	37	" " "
15	♂	35	52	104	52	Control, no exposure
16	♂	35	52	113	61	" "

Summary.

In order to examine the suggestion that ionised air might have the effect of stimulating growth in young rats upon a $-A$ diet, the glass vessels in which they were housed were exposed, when empty, for ten minutes daily to the emanation from radium bromide. The growth of these animals did not differ appreciably from that of animals from the same litters whose homes were not so treated.

CLXXXI. A PRELIMINARY NOTE ON THE GROWTH-PROMOTING AND ANTI-RACHITIC VALUE OF COD LIVER OIL WHEN INJECTED INTRAPERITONEALLY.

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(*Received October 31st, 1924.*)

Attempts have been made by various workers to confer on animals the same protection from a deficiency of the fat-soluble organic factor, by the subcutaneous injections of cod liver oil, as is obtained by its ingestion. Zucker and Matzner [1924] injected the active principle of cod liver oil into rachitic rats and found that no healing was produced; from this they deduced that the active principle must operate through the intestinal tract, thus supporting the theory of Cramer [1923].

That fat can be absorbed when injected either subcutaneously into human beings or intra-tracheally into rabbits has been shown by Schick, Fries, Kohn and Cohen [1924] and by Bossan and Guieysse-Pellissier [1919]; and that the injection of cod liver oil into rats may promote growth, by Wollman and Vagliano [1923]. These authors found cod liver oil and butter fat to be toxic when administered parenterally and only one animal, on a small dosage, survived in the first experiment. The exudate from abscesses was in all cases sterile. I noticed no such effect from the injection of sterile cod liver oil. Wollman and Vagliano conclude from their experiments that fat-soluble A will produce the same effect whether administered by mouth or parenterally, though in the latter case growth is retarded by irregularity of absorption.

Blegvad [1924] injected the active principle of cod liver oil into a patient suffering from night-blindness and observed marked improvement after a fortnight's treatment.

The results of these workers are conflicting and in order to obtain a definite answer to the question as to whether the action of cod liver oil necessarily takes place through the intestinal tract or no, a preliminary experiment on intraperitoneal injections has been carried out.

Experimental.

One series of animals, divided into three groups, was put on McCollum's Diet 3143 [1921]. This is a severe rickets-producing diet.

Group I received Diet 3143 + 0·25 cc. sterile cod liver oil by mouth.
" II " " " " " intraperitoneally.
" III " " without oil, to serve as controls.

The experiment lasted 21–29 days with different litters, and the administration of the cod liver oil was carried out twice weekly during that time. Great care was taken that there should be no oozing of the oil after the injections and the skin was cleaned with light petroleum to remove any trace of oil left by the hypodermic needle. In any case where oozing of oil from the dermic puncture, after severe massage, occurred, the animal was discarded. The oil was sterilised in the autoclave for 1 hour at 120°; none of the animals developed either abscesses or peritonitis.

Post-mortem Findings.

Group I. The rib bones were in all cases firm. The costo-chondral junctions appeared normal except in two cases where a slight cupping was seen.

Group II. Large quantities of non-absorbed oil were found as bright yellow, opaque, beads thickly studding the omentum and, to a lesser degree, the mesentery, peritoneum and fat round the kidneys. The largest masses were hard and firmly adherent to the omentum. These, on microscopic examination, were found to consist of conglomerations of small globules of cod liver oil surrounded by fibrous tissue. The rib bones were somewhat soft and in one or two cases fractures were found and slight angulation at the costo-chondral junctions seen. The general picture in this group was that of moderate rickets.

Group III. All the rats of this group presented an appearance of severe rickets. The bones were soft and in most cases there were multiple fractures of the ribs with angulation at the costo-chondral junctions. Table I gives the histological findings for this series. From this table it will be seen that the

Table I.

No. and sex of rat	Litter	Days on exp.	Diet	Post-mortem findings	Histology	Remarks
Group I						
133 ♀	A	21	3143	Normal	Normal	
134 ♀	A	21	+	,,	,,	
153 ♂	B	29	0·25 cc.	,,	,,	
154 ♂	B	29	oil by	,,	,,	
155 ♂	B	29	mouth	,,	,,	
169 ♀	C	26	,,	,,	,,	
170 ♂	C	26	,,	,,	,,	
Group II						
131	A	21	3143	Rickets +	Rickets + +	No fractures or angulation
132	A	21	+	,, +	,, + + +	,, ,,
152 ♂	B	29	0·25 cc.	,, + +	,, + + + +	Multiple fractures and slight angulation
174 ♀	C	26	oil	,, +	,, + + +	
175 ♀	C	26	injected	,, + +	,, + + +	Angulation, no fractures
Group III						
135 ♀	A	21	3143	Rickets + + + +	Rickets + + + +	Multiple fractures and angulation
136 ♀	A	21	,,	,, + + ±	,, + + + +	
156 ♀	B	29	,,	,, + + +	,, + +	,, ,,
157 ♂	B	29	,,	,, + + ±	,, + + + ±	Angulation, no fractures
158 ♂	B	29	,,	,, + + +	,, + + + ±	Fractures and angulation
171 ♀	C	26	,,	,, + + ±	,, + + + ±	Fractures
172 ♀	C	26	,,	,, + + +	,, + + + +	Multiple fractures and angulation
173 ♂	C	26	,,	,, + + ±	,, + + + ±	Fractures and angulation

protection afforded by intraperitoneal injections of cod liver oil into rats on this diet is slight, but the diet has a very low P and high Ca percentage, and is therefore a drastic one, so that the test is severe and any degree of protection is significant.

A second series of animals was put on a caseinogen diet (F diet), with the following composition:

Inactivated caseinogen	20 g.
Wheat starch	50 ,,
Cotton-seed oil (hardened)	15 ,,
Marmite	5 ,,
Lemon juice	5 cc.
Salt mixture (modified McCollum and Davis, No. 185) ...	5 g.
Distilled water	50 ,,

This diet is deficient in the fat-soluble organic factor. The animals were divided into the same three groups as in Series I and were treated in the same manner. Chart I shows the comparative growth of animals in each group. Each curve represents the average growth for all animals of one litter in that group.

After death, the hind legs (femur, tibia, and fibula) were analysed for calcium content by the method of Aron and Sebauer [1910]. The results are given in Table II.

Table II.

Litter, no. and sex of rat	Diet	Initial age (days)	Final age	Days on diet	Bone analysis: Water %	Bone analysis: Ca % wet	Bone analysis: Ca % dry	Histology
Group I								
D 115 ♀	F +	28	85	57	37·59	12·78	20·47	Normal
D 116 ♂	0·25 cc.	28	85	57	38·96	11·76	19·26	,,
D 117 ♂	oil by	28	85	57	37·33	12·72	20·30	,,
E 142 ♂	mouth	29	85	56	38·88	13·35	21·85	,,
E 143 ♂	,,	29	85	56	42·80	13·43	23·48	,,
E 144 ♀	,,	29	85	56	40·80	12·68	21·43	,,
F 159 ♂	,,	28	87	59	39·76	12·30	20·48	Osteoporosis +
F 160 ♀	,,	28	87	59	38·52	12·51	20·35	Normal
				Av.	39·33	12·69	20·95	
Group II								
D 114 ♀	F +	28	85	57	41·41	11·73	19·52	Normal
E 137 ♂	0·25 cc.	29	85	56	43·63	11·95	21·20	Nearly normal
E 139 ♂	oil in-	29	85	56	43·36	10·58	18·67	Osteomalacia ±
E 140 ♂	jected	29	85	56	43·34	10·94	19·31	Rickets +
E 141 ♀	,,	29	85	56	41·89	12·66	21·75	Normal
E 148 ♂	,,	29	85	56	41·06	11·69	19·84	Nearly normal
F 165 ♂	,,	28	87	59	37·90	11·75	18·92	Normal
F 167 ♀	,,	28	87	59	39·61	11·47	19·00	Osteoporosis ±
				Av.	41·40	11·60	19·77	
Group III								
D 118 ♀	F	28	85	57	46·67	8·32	15·59	Rickets + ±
D 119 ♀	,,	28	85	57	48·07	8·11	15·63	,, + +
D 120 ♀	,,	28	85	57	43·53	7·82	13·84	,, +
E 145 ♂	,,	29	85	56	43·22	10·43	18·37	Normal
E 146 ♀	,,	29	85	56	45·17	8·73	15·90	Rickets +
E 147 ♂	,,	29	85	56	45·92	10·26	18·96	,, +
F 162 ♂	,,	28	87	59	45·63	8·61	16·04	,, + ±
F 163 ♀	,,	28	87	59	Lost	Lost	Lost	Osteomalacia ±
				Av.	45·46	8·89	16·33	

Discussion.

It will be seen that both the growth and calcification of the animals of Group II are distinctly better than those of the controls of Group III, though

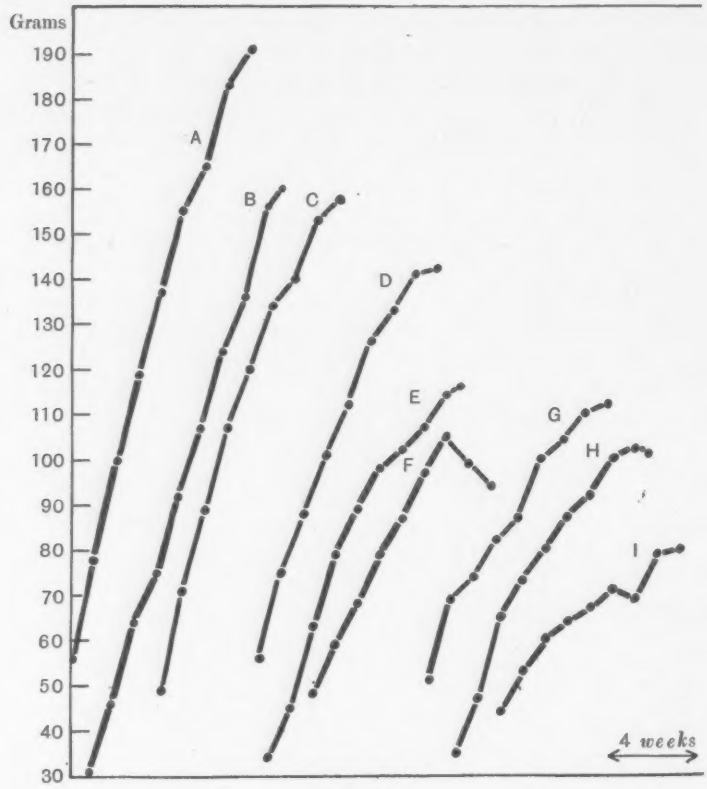

Chart I.

Group I. F diet + 0·25 c.c. cod liver oil by mouth.		Group II. F diet + 0·25 cc. cod liver oil injections.		Group III. F diet (− A).	
A = Nos.	142 ♂	D = Nos.	137 ♂	G = Nos.	145 ♂
	143 ♂		139 ♂		146 ♀
	144 ♀		140 ♂		147 ♂
B =	159 ♂		141 ♀	H =	162 ♂
	160 ♀		148 ♂		163 ♀
C =	115 ♀	E =	165 ♂	I =	118 ♀
	116 ♂		167 ♀		119 ♀
	117 ♂	F =	114 ♀		120 ♀

the values obtained are not equal to those where the oil was administered by mouth.

Intraperitoneal injections were adopted after intramuscular and subcutaneous injections had been found to be unsatisfactory from the point of

view of escape of oil and its possible ingestion by the animal. The injections were limited to two a week. The bulk of the oil was not absorbed, as shown by post-mortem examination, and it is possible that smaller doses administered more frequently would have been better absorbed and thus induced normal growth and calcification.

Summary and Conclusions.

Cod liver oil was injected into rats on:

(1) A rickets-producing diet.

(2) A diet deficient in the fat-soluble organic factor.

Partial protection was afforded against a rickets-producing diet and improved growth and calcification obtained on a diet deficient in the fat-soluble organic factor, thus demonstrating that the site of action of cod liver oil is not limited to the intestinal tract.

I have much pleasure in thanking Professor C. J. Martin and Professor V. Korenchevsky for the large measure of help and advice given to me by them throughout the experiment. I have also to acknowledge my indebtedness to the Medical Research Council for a whole time grant and to the Lister Institute for the hospitality of its laboratories.

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CLXXXII. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

PART V. THE ENZYME IN THE EARLY STAGES OF BONE DEVELOPMENT.

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(*Received November 1st, 1924.*)

Previous experiments [Robison, 1923], which demonstrated the presence of a phosphoric esterase in the bones of young animals, failed to detect this enzyme in unossified cartilage. The present work was undertaken to confirm this observation, and to determine whether any correlation could be traced between the appearance of the enzyme and the beginning of the ossification process in the cartilage.

Our first experiments were carried out on the cartilages of a full-term infant who died 43 hours after birth. Extracts were made (*a*) from the costochondral junctions, including half-an-inch of bone and of cartilage on either side of the line of ossification, (*b*) from the costal cartilages at their sternal ends, and (*c*) from the patellae. In (*b*) and (*c*) no sign of commencing ossification could be made out.

The parts to be examined were dissected free from muscle or ligament, cut into thin slices, and ground up with twenty times their weight of distilled water saturated with chloroform, in which they were allowed to remain for 48 hours at room temperature. These 5 % extracts were then tested, without filtration, for the presence of the enzyme, using sodium glycerophosphate as the substrate. A number of tubes was prepared, each containing

10·0 cc. *M*/1000 sodium glycerophosphate solution.
1·0 „ 5 % tissue extract.
0·1 „ phenolphthalein, alkali, etc., to adjust the p_H to 8·4.
0·1 „ chloroform.

These were kept in a thermostat at 38° and at the stated intervals a tube was removed and the inorganic phosphate estimated by the Briggs method after precipitation of the proteins with trichloroacetic acid. The results as shown in the tables give the amount of hydrolysis effected by 1 cc. of the 5 % extracts. Control tubes containing tissue extract + water, and substrate + water, were simultaneously examined, but as these showed no increases in inorganic phosphate the figures have been omitted from the tables.

Table I. *Cartilages of full-term infant.*

Source of 5 % extract.	Hydrolysis of $M/1000$ sodium glycerophosphate (10 cc. = 0·31 mg. P.) effected by 1 cc. 5 % extract at 38°, p_H 8·4. mg. P.	
	3 hours	18 hours
Costochondral junction	0·066	0·182
Costal cartilage, unossified	0·006	0·006[1]
Patella, unossified	0·009	0·009[1]
Bones of young rats (control)	0·106	0·264

We next investigated the bones of a human foetus of $5\frac{1}{2}$–6 months' development. The femora, tibiae and humeri were dissected out and divided into three portions, (*a*) completely ossified shaft, (*b*) the epiphyseal cartilages in which no ossification was apparent, and (*c*) the junction between (*a*) and (*b*) including the line of ossification. In the case of the metatarsals and of the pelvic bones the separate centres of ossification were dissected as completely as possible from the unossified cartilage. Extracts were made of these various parts and tested in the manner detailed above, except that in this case the concentration of the sodium glycerophosphate was $M/100$. An extract of the parietal as an example of a membrane bone was also prepared and tested.

Table II. *Skeleton of a $5\frac{1}{2}$ months foetus.*

Source of 5 % extract.		Hydrolysis of $M/100$ sodium glycerophosphate (10 cc. = 3·1 mg. P.) effected by 1 cc. 5 % extract at 38°, p_H 8·4 mg. P. in 18 hours
Femur	Shaft (ossified) ...	1·85
	Junction	2·20
	Epiphysis (unossified)	0·010
Tibia	Shaft (ossified) ...	1·46
	Junction	1·74
	Epiphysis (unossified)	0·013
Humerus	Shaft (ossified) + Junction ...	2·08
	Epiphysis (unossified)	0·006
Metatarsals	Ossifying centres ...	0·303
	Cartilage (unossified)?	0·095
Ileum, Ischium, Pubis	Ossifying centres ...	1·76
	Cartilage (unossified)	0·006
Parietal	Ossified	1·28

Both these experiments show that while the ossified portions of the young bones contained the phosphoric esterase in a high degree, the enzyme was not present in significant amount in the epiphyses of the long bones, in which the ossification centres had not yet appeared, nor in the costal cartilages, nor in the unossified cartilage of the pelvic bones. The patella which alone was entirely cartilaginous also contained none of the phosphoric esterase.

[1] The identity of these figures with those for 3 hours suggests that this small increase in the inorganic phosphate was not due to enzymic hydrolysis of the ester.

The metatarsal cartilages in Exp. 1 appeared at first to give an exceptional result, but a close examination of the parts[1] from which the extract had been made showed that some of the minute adjacent bones (phalanges), which contained ossifying centres, had been included and would account for the enzymic activity of the extract. It is interesting also to note the presence of the enzyme in membrane bones (parietals) as well as in those developed from cartilage.

The evidence of these experiments is sufficient proof that in partially ossified bones the enzyme is present only in those parts where active ossification is proceeding. We wished to carry the argument a stage further and to show that in one and the same bone, the first appearance of the enzyme coincides with the appearance of the ossification centre. We were here met by the difficulty of obtaining a human foetus at a sufficiently early stage of development for our purpose. The centres of ossification appear, for the most part throughout the human skeleton, during the second and third months of foetal life, and bone formation proceeds with such rapidity that within the course of a few weeks the greater part of the shafts of the long bones becomes calcified. The earliest foetus we succeeded in obtaining, of about $2\frac{1}{2}$–3 months' development already showed extensive ossification throughout the long bones and ribs. We therefore sought for some bone which might be obtained in an entirely unossified state. This requirement is fulfilled by the patella, which remains entirely cartilaginous until three years or more after birth, when a single centre of ossification appears, from which bone formation spreads slowly, becoming complete at about puberty. We have compared the hydrolytic activity of cartilaginous and ossifying patellae obtained post mortem from children of varying ages. The following notes give details of the material dealt with and the presence or absence of phosphoric esterase, the experimental figures being shown in Table III.

A. 5 % extract of patellae from two still-born children; both entirely unossified. No phosphoric esterase was present.

B. 5 % extract of patella from a 7 months old child; entirely unossified. No phosphoric esterase was present.

C. 5 % extract of patella from a boy aged 5 years. The patella was bisected antero-posteriorly in the vertical plane, and the half from which the extract was made showed no ossification. No phosphoric esterase was present. The other half, kept for histological examination, was subsequently found to contain a well-developed centre of ossification, eccentric in position. As this ossifying half had been soaked in formalin for two days, the presence of an enzyme was not to be expected, nor was any found in the extract made therefrom.

D. 5 % extract of patella from a girl aged 6 years. The patella was bisected

[1] Fortunately in this case, owing to the minute amount of the tissues, these had not been macerated but merely allowed to soak in the water. It was thus possible to detect the phalanges with the aid of a lens.

antero-posteriorly in the vertical plane. The cut surface, which measured 32×16 mm. showed a well-developed centre of ossification, central in position and oval in shape, measuring 10×5 mm. Early ossification was also seen just deep to the periosteum, extending radially towards the centre of the bone. The extract was prepared from one half of the patella and although this included a large proportion of cartilage the extract showed a very considerable enzymic activity.

Table III.

Hydrolysis of $M/100$ sodium glycerophosphate effected by 1 cc. 5 % extract at 38°, p_H 8·4.

Source of 5 % extract.	mg. P. 3 hours	mg. P. 18 hours
A. Unossified	0·000	0·000
B. Unossified	0·000	0·000
C. Unossified half	—	0·002
D. Partly ossified	0·035	0·220

We have thus clear proof that prior to the appearance of the centre of ossification the patella does not contain the phosphoric esterase but that this enzyme is present soon after ossification has commenced.

Summary.

An investigation of cartilages, and of bones in early stages of development, from human embryos and young infants has shown that the enzyme which hydrolyses monophosphoric esters is not present in any cartilage prior to the appearance of a centre of ossification but is found wherever active ossification is taking place.

It therefore seems legitimate to conclude that the two phenomena are bound up together and that the production of the enzyme is a part of those cellular activities which result in the formation of bone.

We are greatly indebted to Dr R. T. Brain of the London Hospital and to Dr E. H. Lepper of the Elizabeth Garrett Anderson Hospital for their help in supplying the material for these experiments.

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CLXXXIII. THE HYDROGEN ION CONCENTRATION OF THE WHOLE BLOOD OF NORMAL MALES AND OF CANCER PATIENTS MEASURED BY MEANS OF THE QUINHYDRONE ELECTRODE[1].

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(*Received November 3rd, 1924.*)

The present paper contains an account of measurements of the hydrogen ion concentration (or the p_H) of whole blood by means of the quinhydrone electrode, an electrode introduced for the purpose of the measurements of hydrogen ion concentrations by Biilmann [1921]. Up to the present, all direct measurements of the p_H of blood have been carried out by means of either the indicator, or the hydrogen gas electrode methods. The indicator method is only applicable to blood plasma, and inaccuracies are liable to be encountered on account of the necessity of separating the plasma from the corpuscles. The hydrogen gas electrode method is cumbersome, both from the point of view of time, and also on account of the fact that elaborate precautions have to be adopted in order to prevent the loss of carbon dioxide.

The quinhydrone electrode, on the other hand, promised to be simpler, more rapid in action, and consequently convenient for the purpose in view, namely, a comparison of the p_H of the whole blood of normal and of cancer patients, respectively.

The quinhydrone electrode is essentially an oxidation-reduction electrode and is based on the equilibrium set up in aqueous solution between quinone and quinol, namely,

$$C_6H_4O_2H_2 \rightleftarrows C_6H_4O_2 + 2H^+ + 2\theta.$$

This equilibrium may be represented by the equation

$$\pi = \pi_0 + \frac{RT}{2F} \log \frac{[\text{Quinone}]}{[\text{Quinol}]} + \frac{RT}{F} \log H^+,$$

where π is the observed potential of such an electrode, and π_0 is that when the hydrogen ion concentration (or activity) is unity and the molecular ratio of

[1] This investigation was undertaken on behalf of the Liverpool Cancer Research Committee. Director: Professor W. Blair Bell of the University of Liverpool.

quinone and quinol is unity. The numerical value of π_0 is 0·9802 volt at 37°. In order to ensure that the molecular ratio of quinone and quinol is unity, the compound quinhydrone is employed. A solution of quinhydrone in water is widely dissociated to give equimolecular proportions of quinone and quinol.

Biilmann [1923] has shown that the values of the p_H of buffer solutions, measured by the quinhydrone electrode, agree with those measured by means of the hydrogen gas electrode, and this has been confirmed during the present work. Further it has been found during this investigation, that the quinhydrone electrode gives correct values for the p_H of solutions of lead salts. It has previously been shown by Denham and Allmand [1908] that the hydrogen gas electrode fails to function in solutions of lead acetate, owing to the reduction of bivalent lead ion to monovalent lead ion by hydrogen gas, with a corresponding decrease in the hydrogen pressure at the electrode. Thus in the case of lead salts, the quinhydrone electrode gives correct values for the p_H when the hydrogen gas electrode fails to function.

The Hydrogen ion Content of Blood.

In the measurement of the p_H of blood, the main cause of error lies in the fact that blood rapidly loses carbon dioxide, thereby impairing the efficiency of the bicarbonate system with the result that the blood becomes more alkaline. In view of this factor, rapid measurements were aimed at in the present investigation, the blood coming into contact with the air for the shortest possible time.

With reference to the use of quinhydrone, this substance itself might react with the blood (for example, interaction between quinone and haemoglobin to form methaemoglobin) and so cause erroneous results. It will be shown later, however, that such a reaction, should it occur, is comparatively slow.

The commonly accepted value for the p_H of blood, as measured by indicators (use of indicators is limited to the case of blood plasma only) and by the hydrogen gas electrode, is 7·4–7·5. Hasselbalch and Lundsgaard [1912], however, found by means of the hydrogen gas electrode that the p_H of defibrinated blood is about 7·4 and this value lies between those of the p_H of blood serum (7·8) and of red blood corpuscles (7·0). In the following work, the p_H of whole blood (not defibrinated blood) has been measured in every case.

Experimental.

For the purpose of the measurement of the p_H of blood, a small vessel of about 2 cc. capacity was employed. The vessel had a narrow side limb and could be completely filled with blood. In the first place it must be stated that when a *platinum* foil electrode was employed in conjunction with the quinhydrone, unreasonable values (such as $p_H = 13$) were obtained for the p_H of sheep's whole blood. Since the platinum foil functions other than as an

inert electrode (for example, as a catalyst in the oxidation of quinol), gold foil was substituted[1].

The E.M.F. of the following cell was measured at 37°:

Hg | Hg_2Cl_2 | KCl (N) | saturated KCl | blood saturated with quinhydrone | Au.

The value of the normal calomel electrode at the temperature of the experiment was taken as 0·5731 volt, whilst RT/F was 0·0615. The formula from which the results were calculated was the following:

$$p_H = \frac{0{\cdot}9802 - (0{\cdot}5731 + E)}{0{\cdot}0615},$$

E being the observed electromotive force of the above cell, its value being positive when the gold electrode was the positive pole of the cell.

Preliminary experiments, employing the gold-quinhydrone electrode, showed that values for the p_H of blood of the right order of magnitude could be obtained. It was concluded, therefore, that any reaction between the blood and the quinhydrone, in the presence of gold foil, was very slow. In these preliminary approximate experiments, however, the values, although of the correct order of magnitude, were not reproducible enough to render the method reliable as it stood.

The following technique for the measurement of the p_H of blood was eventually evolved. About 3 cc. of blood were collected rapidly from a vein (in the case of humans, of the arm) through a large hollow needle and shaken vigorously in a small vessel with a little quinhydrone for about ten seconds. Vigorous shaking was necessary, as the quinhydrone was not readily wetted by blood. The blood was then rapidly transferred to the electrode vessel and forced through the side limb by pressing down the rubber stopper containing the gold foil electrode. The blood, which was still warm and unclotted, attained the temperature of 37° in a few seconds and the reading was taken immediately. Alternately, time readings of the potential were taken and the readings were extrapolated to zero time. By the latter means, any variation due to slow chemical interaction could be detected. In general, however, the potential drift with time was so slow, that the reading obtained within a minute of the introduction of the quinhydrone into the blood could be taken as the correct one.

The above technique was followed in all of the subsequent work. The advantages are (*a*) a considerable saving of time as compared with other methods, (*b*) that the p_H of whole blood was obtained, since the blood was introduced into the electrode vessel before it had clotted. The absence of any sudden change in the E.M.F. during clotting indicated that the process of clotting did not affect the value of the p_H.

After each measurement, it was found essential to clean the gold electrode

[1] Whilst this work was in progress, a paper by Dixon and Quastel [1923] appeared in which it was stated that the use of gold was preferable to that of platinum in the measurement of reduction potentials, in this case of cystine-cysteine systems.

with chromic acid, followed by boiling in distilled water. Before a measurement, the electrode was tested by means of buffer solutions of known p_H.

Results.

(i) *Values of the p_H of whole human blood.* In the first place, normal human subjects were examined. From the results given in Table I, it will be seen that the average value at 37° is 7·33.

It may be mentioned that the average value obtained for human defibrinated blood at 37° by Schade, Neukirch and Halpert [1921] by means of the platinum-hydrogen electrode, employing a mixture of hydrogen and carbon dioxide, is 7·31.

Table I. *Values of the p_H of the whole blood of normal males at* 37°.

Exp.	p_H	Exp.	p_H
1	7·33	6	7·30
2	7·37	7	7·33
3	7·36	8	7·33
4	7·33	9	7·33
5	7·33		

Average value = 7·33.

(ii) *Values of the p_H of the blood of cancer patients.* In Table II are given the results of measurements of the p_H of the blood of cancer patients, together with details of any treatment received prior to the measurement of p_H.

It is evident from the results shown in Table II that there is no marked difference between the p_H value of the blood of cancer patients and that of normal human blood, the average values being 7·32 and 7·33 respectively. The extreme values obtained in the case of cancer patients, namely 7·19–7·45, lay further apart than those obtained for normal blood. The absence of any

Table II. *Values of the p_H of the blood of cancer patients at* 37°.

Case no.	Sex	Diagnosis	p_H	Previous treatment
5	Female	Carcin. breast	7·35	X-ray treatment
6	,,	Carcin. both breasts	7·39	X-ray treatment
7	,,	Carcin. cervix	7·30	Operation
8	,,	Carcin. stomach	7·37	None
10	,,	Carcin. thyroid	7·39	None
13	Male	Sarc. stomach	7·45	Operation
16	,,	Sarc. neck	7·35	X-ray treatment
17	Female	Carcin. cervix	7·21	Operation
18	,,	Carcin. colon	7·39	
19	,,	Carcin. rectum	7·20	
21	Male	Carcin. parotid	7·33	Radium treatment
22	Female	Recurrent carcin. of breast	7·37	Operation and X-ray treatment
23	,,	Carcin. rectum	7·31	None
24	,,	Carcin. breast	7·30	Operation (2 years previously) Radium (1 year previously)
25	,,	Recurrence after breast amputation	7·27	
26	Male	Sarcoma	7·20	Deep X-rays
27	Female	Carcin. uterus	7·19	Vaginal hysterectomy Three years' history of haemorrhage
		Average value	7·32	

appreciable difference in the p_H of the blood of normal and cancerous subjects is in agreement with results obtained by Woglom [1924], using the hydrogen gas electrode method of measurement. It may be stated generally that immediately following an operation, the p_H is lowered. This may be due either to the products of autolysis of damaged tissues, or to the effects of the enforced physical inactivity following an operation.

(iii) *The effect of intravenous injection of lead suspension (electrically prepared)*[1] *on the p_H of the blood of cancer patients (in connection with the action of lead on cancerous growths)* [cf. Blair Bell, 1922, 1924]. It was of importance to determine the effects of injections of lead suspension on the p_H of the blood. The concentration of the suspension (of which various amounts were given) was 0·1 % in respect of lead. The suspension itself was made slightly hypertonic, containing, in addition to lead and gelatin, 2 % sodium chloride, 0·05 % calcium chloride, and 0·05 % potassium chloride.

In Table III are summarised a group of results which suggest generally that treatment with lead suspension, injected intravenously, is accompanied by a fall in the p_H of the blood of cancer patients, that is, the blood becomes slightly more acid[2].

Table III. *The effect of lead injections on the p_H of the blood of cancer patients.*

Case	Date	p_H before treatment	Treatment Total amount Pb g.	Date	p_H after treatment
5	23. iv. 24	7·33	0·095	4. vii. 24	7·24
6	5. v. 24	7·39	0·01*	19. v. 24	7·24
7	14. v. 24	7·30	0·126	17. vi. 24	7·23
10	13. vi. 24	7·39	0·154	22. vii. 24	7·27
13	1. vii. 24	7·45	0·06	11. vii. 24	7·30
17	9. vii. 24	7·21	0·15	24. vii. 24	7·22
21	22. vii. 24	7·33	0·18	15. viii. 24	7·30
24	30. vii. 24	7·20	0·21	26. viii. 24	7·16
	Average value	7·32		Average value	7·24

* This patient underwent an operation immediately after the first measurement of p_H (*i.e.* before lead treatment had commenced).

Having obtained the general result that the effect of injection of lead is to lower the p_H of the blood, it was next necessary to determine whether such injections exerted a permanent depressing effect, or whether, between injections, the p_H tended to attain its original value. Measurements before and after injections carried out over a considerable period of time, in the case of five patients, showed that the p_H tends to rise to its original value. It was also found that the injection of a solution containing those substances, other

[1] This material is obtained by sparking lead electrodes under water containing calcium chloride and gelatin, as stabilising agents. By proper adjustment of conditions a suspension consisting of metallic lead together with hydroxide, and possibly a trace of carbonate, can be obtained in which the size of particles does not exceed 0·3 μ.

[2] It has been shown by La Barre [1924] that injections of electrargol cause a lowering of the p_H value of the blood plasma of guinea-pigs. This result is analogous to the above.

than the lead, which were present in the lead suspension (*i.e.* gelatin, sodium, potassium, and calcium chlorides) has practically no effect on the p_H.

Summary.

1. The measurement of the p_H of whole blood by means of the quinhydrone electrode has been described. As platinum foil has been found to be unsatisfactory when employed as electrode metal in the case of blood, a gold foil electrode was used instead, with satisfactory results.

2. By this means it has been found that the average value for the p_H of normal human subjects is 7·33. It was also found that the p_H of the blood of cancer patients is identical, within the limits of error, with that of the blood of normal human subjects.

3. The effect of intravenous injection of a lead suspensoid, prepared by the Bredig sparking method, is to lower the p_H immediately following such injections; but the lowering is only temporary.

The authors wish to express their thanks to Mr W. R. Williams, F.R.C.S., for help in the clinical side of this investigation.

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CLXXXIV. LECITHIN AND CHOLESTEROL IN RELATION TO THE PHYSICAL NATURE OF CELL MEMBRANES[1].

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(*Received November 3rd, 1924.*)

Although the chemical problems presented by cell membranes are still obscure, work carried out by Clowes [1916] on the reversal of oil-water emulsions by ions, and the application of the results of this work to permeability phenomena in biology, have lent support to the view that cytoplasm consists essentially of an emulsion system containing fatty material and water. Working with olive oil-water systems *in vitro*, Clowes has shown that electronegative ions, such as hydroxyl ion, favour the formation of a system in which water is the continuous phase and oil the disperse phase, whilst electropositive ions, such as calcium ion, favour the reverse type of system. When the aqueous phase consists of a mixture of sodium hydroxide and calcium chloride, the water is the continuous phase provided that the sodium hydroxide is present in excess of the molecular ratio, 4 of NaOH to 1 of $CaCl_2$, whilst the oil is the continuous phase if the calcium chloride is present in excess of this ratio. The fact that systems in which oil is the continuous phase are much less permeable to water-soluble substances than are systems in which water is the continuous phase, was the basis upon which Clowes formulated his conclusions regarding the permeability of cell membranes, viewed in the light of his own results.

It is with the object of examining the antagonistic action, if any, of the substances lecithin[2] and cholesterol in relation to permeability that the following work has been carried out. The ultimate object is to attempt to account in some degree for the increased permeability to water-soluble substances attributed to cancer cells.

The general method of procedure has been to investigate the emulsifying powers of these two substances on typical fatty oil-water systems.

[1] This investigation was undertaken on behalf of the Liverpool Cancer Research Committee. Director: Professor W. Blair Bell of the University of Liverpool.

[2] In general, the lecithin employed was a commercial lecithin which contained pure lecithin, kephalin, sphingomyelin, but no cholesterol.

THE EFFECT OF CHOLESTEROL AND LECITHIN, RESPECTIVELY, ON THE INTERFACIAL TENSION BETWEEN OIL (OLIVE OR COTTONSEED) AND WATER.

In the first place, the effect of cholesterol, dissolved in the oil phase, on the interfacial tension between oil and water, was compared with that of calcium chloride, present in the aqueous phase. The drop pipette method was employed. The results of this comparison are shown in Tables I and II below. The interfacial tensions were calculated from the corresponding drop numbers, the latter quantities being inversely proportional to the interfacial tensions, provided that the densities of the two phases do not sensibly change. The value for the interfacial tension between pure olive oil and water was taken as 18·6 [cf. Quincke, 1870]. The concentration of cholesterol (and, incidentally, of lecithin) in the following work is expressed as a percentage, that is, as the number of grams per 100 cc. of the phase in which it is present (either oil or water).

Table I. *Drop numbers and interfacial tensions of olive and cottonseed oils, respectively, in water in the presence of cholesterol, dissolved in the oil phase.*

	Olive oil		Cottonseed oil	
% cholesterol	Drop number	Interfacial tension dynes/cm.	Drop number	Interfacial tension dynes/cm.
0·0	72	18·6	66	20·3
0·5	—	—	67	20·0
1·0	89	15·0	76	17·6
1·25	—	—	83	16·1
2·0	117	11·4	—	—
2·5	130	10·3	—	—

Table II. *Drop numbers and interfacial tensions of olive and cottonseed oils, respectively, in contact with aqueous solutions containing calcium chloride.*

	Olive oil		Cottonseed oil	
% calcium chloride	Drop number	Interfacial tension dynes/cm.	Drop number	Interfacial tension dynes/cm.
0·0	72	18·6	66	20·3
0·1	73	18·5	—	—
0·25	95	14·1	66	20·3
0·5	180	7·4	69	19·4
2·0	—	—	75	17·9
4·0	—	—	106	12·6

The above results show that cholesterol and calcium chloride have a similar effect on the interfacial tension between oil and water, in that they cause a decrease. In the case of olive oil and water, the interfacial tension is lowered to a greater extent by calcium chloride than by cholesterol, whilst in that of cottonseed oil and water, cholesterol has the larger effect. Presumably this is due to the fact that the cottonseed oil contained less fatty acids than did the olive oil and consequently there was less tendency on the part of the calcium chloride towards the formation of a soap. Donnan [1899] has shown

that substances such as sodium hydroxide have only a very small effect on the interfacial tension between pure oils, containing no fatty acids, and water. The same would be true in the case of calcium hydroxide or a calcium salt, since the effects of sodium and calcium salts on such interfacial tensions depend upon the formation of a soap. The action of cholesterol, on the other hand, does not depend upon the formation of a soap.

From Clowes' work, it is known that the presence of calcium chloride favours the water-dispersed-in-oil type of emulsion. It does not follow from the above interfacial tension measurements alone that cholesterol favours this type. However, it is fairly generally recognised [cf. Hughes and King, 1923] that any substance, soluble in a fat phase, which lowers the interfacial tension in a fatty oil-water system will favour an emulsion of the water-dispersed-in-oil type. Cholesterol is such a substance and hence one would expect it to form a water-dispersed-in-oil emulsion. That such is the case has been shown by Seifriz [1923] and this has been confirmed during the present work.

An attempt was also made to study the effect of lecithin on the interfacial tension between oil and water, olive oil only in this case being employed. Lecithin is practically insoluble in olive oil and in water, but disperses readily in the latter.

In the first place, olive oil was saturated with lecithin, and the interfacial tension between such a solution and water was measured by the drop pipette method. When the sample of lecithin was entirely free from cholesterol, there was no definite effect on the interfacial tension, owing presumably to the negligibly small solubility of such lecithin in olive oil. When a sample of lecithin containing cholesterol was employed, the increase in the drop number of the olive oil in water was quite marked and corresponded to a decrease in the interfacial tension between the oil and water of 10 %. From the results obtained for the interfacial tensions of olive oil in water, in the presence of cholesterol, it may be shown that the amount of cholesterol, dissolved in the oil, which would cause such a decrease, is 0·5 %. But the total dissolved lecithin was much less than 0·5 % and therefore the amount of cholesterol must have been less than 0·005 %. Hence we infer that the lecithin, in so far as it is dissolved in the oil, whether in the form of a lecithin-cholesterol complex or not, behaves in a similar manner to cholesterol, in lowering the interfacial tension between olive oil and water. These measurements do not give any information regarding the type of emulsion formed between water and oil when lecithin is used as emulsifying agent. However, previous work [*vide* Bhatnagar, 1921; Seifriz, 1923] has shown that lecithin as emulsifying agent, favours an oil-dispersed-in-water system.

In studying the effects of the presence of both lecithin and cholesterol on the interfacial tension, the lecithin, as already mentioned, was dispersed, not dissolved, in the aqueous phase; the cholesterol, on the other hand, was dissolved in the oil phase.

The general result obtained was that, although these substances give rise

to different types of emulsion systems, their combined effect on the interfacial tension between oil and water is additive. The following table gives the results of such measurements.

Table III. *Drop numbers and interfacial tensions of olive oil in water in the presence of a mixture of lecithin and cholesterol.*

% cholesterol in the oil phase	% lecithin in aqueous phase	Drop number	Interfacial tension dynes/cm.
0·0	0·0	72½	18·6
0·0	1·0	91	14·8
0·5	1·0	95	14·2
1·0	1·0	105½	12·8
1·5	1·0	121	11·1

The antagonistic effects of Lecithin and Cholesterol on oil-water systems.

In order to ascertain whether lecithin (cholesterol-free) and cholesterol itself, exert antagonistic effects when employed together as emulsifying agents in oil-water systems, it was of importance to examine the stability and nature of the emulsions formed by the individual substances. In all of the following work, the types of the emulsions were determined by means of the Briggs drop method, that is, by adding a drop of the emulsion to water and so discovering whether oil or water was the continuous phase. Such results were also confirmed in most cases by staining one phase and examining microscopically. Attention was paid to the ease with which the emulsions were formed in the respective cases.

When equal volumes of a 1 % aqueous lecithin dispersion and olive oil were shaken, complete emulsification occurred after about half-a-dozen shakes. Such an emulsion was of the oil-dispersed-in-water type and was fairly stable, a cream only separating out after several hours.

However, when a solution of 1 % cholesterol in olive oil was shaken with an equal volume of water, complete emulsification was not attained with the same ease. Intermittent shaking was resorted to, that is, the mixture was allowed to stand for about half-a-minute between each half-a-dozen shakes. After thirty-five to forty shakes, complete emulsification occurred and the emulsion was of the water-dispersed-in-oil type.

On shaking a 0·02 % aqueous cholesterol dispersion[1], with an equal volume of pure olive oil, complete emulsification occurred after about fifty shakes, the emulsion being of the water-dispersed-in-oil type. This observation agrees with that of Seifriz [1923], who employed equal volumes of a 0·01 % aqueous cholesterol dispersion and olive oil.

The emulsions formed through the emulsifying agency of cholesterol were not as stable as those formed in the case of lecithin. Breaking down commenced immediately the emulsions were allowed to stand and after half-an-hour, practically complete separation of the two phases had occurred.

In view of the totally different types of emulsion formed by lecithin and

[1] Prepared by adding 1 cc. of a 2 % solution of cholesterol in alcohol to 99 cc. of water.

cholesterol, respectively, especially in respect of stability, caution had to be exercised in the later determinations of the type of emulsion which resulted when mixtures of lecithin and cholesterol were employed as emulsifying agents. For example, in practically every case in which the two emulsifying agents were present, the emulsion formed after the first dozen shakes was of the oil-dispersed-in-water type. In those cases in which reversal of phase did actually occur, such reversal was only attained after a great deal of intermittent shaking. In one case, reversal took place after about six hours' shaking, but at the end of this time the emulsion which, after the first few shakes, was of the oil-dispersed-in-water type, undoubtedly had become reversed to the water-dispersed-in-oil type.

The reason for the above phenomenon probably lies in the differences in the ease with which emulsification is brought about by lecithin and cholesterol, respectively.

In Table IV appear the results of experiments carried out on the above lines, employing mixtures of lecithin and cholesterol as emulsifying agents, both of these substances being originally present in the aqueous phase. Concentrations are expressed as percentages, as before.

Table IV. *The effect of mixtures of lecithin and cholesterol on emulsion systems.*

Vol. of oil phase cc.	Vol. of aqueous phase cc.	% lecithin in aqueous phase	% cholesterol in aqueous phase	Ratio lec./chol.	Continuous phase
5	6·0	0·33	0·017	19·4	Water
5	5·5	0·18	0·018	10·0	,,
5	5·4	0·148	0·0185	8·0	(Indefinite)
5	5·3	0·113	0·019	6·0	Oil
5	5·2	0·077	0·019	4·1	,,
5	5·1	0·04	0·02	2·0	,,

From the results shown in Table IV, for the case in which lecithin and cholesterol were both present in the aqueous phase, it is evident that the point of inversion of the emulsion occurs when the ratio of the concentration of lecithin to that of cholesterol, expressed in g. per 100 cc. aqueous solution, is about 8:1.

When, however, the cholesterol was originally present in the oil phase and the lecithin in the aqueous phase, relatively less lecithin was required at the point of inversion of the emulsions, as is shown by the results given in Table V.

Table V. *The effect of mixtures of lecithin, present in the aqueous phase, and cholesterol, in the oil phase, on emulsion systems.*

Vol. of oil phase cc.	Vol. of aqueous phase cc.	% lecithin in aqueous phase	% cholesterol in oil phase	Lec./chol. ratio	Continuous phase
5	5	0·08	1·0	0·08	Oil
5	5	0·2	1·0	0·2	,,
5	5	0·4	1·0	0·4	,,
5	5	1·0	1·0	1·0	(Indefinite)
5	5	2·0	1·0	2·0	Water

From the results shown in Table V, it is concluded that the ratio of the concentration of lecithin to that of cholesterol at the inversion point is between 1:1 and 2:1, the cholesterol being originally present in the oil phase. In the case in which cholesterol was originally present in the aqueous phase (in the dispersed form), the ratio of the concentrations of lecithin to cholesterol at the inversion point was 8:1[1].

Bearing in mind that, in the body, lecithin and cholesterol are most probably present in that phase in which they are more readily soluble, namely, aqueous and fatty phases, respectively, it is presumed that the results quoted in Table V will be more applicable to the permeability phenomena exhibited by cell membranes, than will those which appear in Table IV.

Discussion of Results.

As previously mentioned, the foregoing measurements were carried out with the intention of attempting to account for the increased permeability of cancerous tissues, compared with that of normal tissues. Assuming that cytoplasm consists, even if only partly, of an emulsion formed between fats and water, it is evident that the conclusions arrived at by means of experiments carried out *in vitro*, such as those described, should be applicable to the definition of the state of such cytoplasm as a function of, for example, its lecithin and cholesterol content. Whatever other rôle these two substances may have, such as the regulation of oxidation processes in the cells, it is of primary importance to consider their part in the problem of the permeability of cell membranes, since changes in the permeability of an emulsion system such as is represented by cell membranes will most certainly affect the normal metabolism of the cell by modifying the direction and extent of ferment action. Thus it is conceivable that changes in permeability may stand in intimate relation to various abnormal states of the tissues, for example, neoplasia. If the lecithin-cholesterol ratio were found to be higher in cancer cells than in normal cells, it would be expected, on the basis of the above results, that cancer cells would be more permeable to water-soluble substances than would normal cells. A few results obtained by Bullock and Cramer [1913] on the lecithin and cholesterol content of malignant tumours, indicate that the lecithin-cholesterol ratio is higher in rapidly growing tumours than in slowly growing tumours. Hence in the light of the results detailed in the present paper, one would expect that rapidly growing tumours would be more permeable to water-soluble substances than would slowly growing tumours. It has actually been shown by Waterman [1922] that cancerous tissues, freshly removed from the body, have a higher electrical conductivity and permeability, than normal tissues under the same conditions.

[1] It has been suggested by Professor Ramsden that the differences in inversion points, indicated by Tables IV and V, respectively, may possibly be due to two different kinds of complex formed between lecithin and cholesterol, the constitution of the complex depending on the conditions which attend its formation.

It is not suggested that the lecithin-cholesterol ratio is the only important factor in determining the permeability of cancer tissues. It has been found that rapidly growing cancer cells are deficient in calcium and rich in potassium [Clowes and Frisbie, 1905]. This latter condition would also point to increased permeability of such cells.

Mayer and Schaeffer [1914] have already suggested that the state of protoplasmic membranes is dependent upon the relative amounts of lecithin and cholesterol present, but they ascribe the effect of these substances to an alteration in the degree of dispersion of the fats contained in cytoplasm.

Summary.

1. The effect of lecithin, cholesterol, and mixtures of these two substances on the interfacial tension between oil and water has been studied. Both substances cause a lowering of the interfacial tension, and in the case of mixtures of the two substances, the effect is additive, in spite of the fact that lecithin and cholesterol favour different types of emulsion.

2. Emulsification experiments on olive oil-water systems have been carried out, in order to examine the antagonistic effect, if any, of lecithin and cholesterol on such systems. In agreement with the work of Seifriz [1923], lecithin is found to favour an oil-dispersed-in-water type of emulsion, whilst cholesterol favours the reverse type. When mixtures of these substances were present in the aqueous phase, antagonistic effects on the type of emulsion were observed. The inversion point of the emulsion occurred when the ratio of the concentrations (expressed in g.) of lecithin to cholesterol was about 8: 1, both substances being present initially in the aqueous phase. When cholesterol was present in the oil phase to begin with, and the lecithin was in the aqueous phase, the inversion ratio was found to be between 1 : 1 and 2 : 1. Probably the latter condition, namely, that in which the lecithin and cholesterol are dissolved in different phases, approximates more closely to the conditions found in cell membranes. Consequently the latter result, which states that inversion takes place when the ratio of lecithin to cholesterol is between 1:1 and 2:1, is probably the more significant result in connection with permeability phenomena, exhibited by cell membranes[1].

[1] The above ratio has been found by Lange and Lawaczeck [1923] to be that at which the rate of oxygen intake is a maximum in the case of the system—lecithin, cholesterol, ferric cloride, water, *in vitro*.

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CLXXXV. STUDIES ON INOSITOL.

III. THE METABOLIC BEHAVIOUR OF *i*-INOSITOL IN THE DEVELOPING AVIAN EGG.

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In the previous communications of this series [Needham, 1923, 1, 2, 1924], experiments were described which were planned to throw some light on the physiological significance of *i*-inositol. In the first paper an estimation method for this substance was described, and in the second it was shown that the animal body appeared to possess the powers of effecting its synthesis. The present paper describes some work which has been done to determine what changes inositol may undergo during embryogenesis. In view of the correlation of the cycloses with the growth process by Starkenstein [1911] such an undertaking was the more desirable.

The importance of inositol in the egg was partially realised by two of the earlier observers. Rosenberger [1908] was unable to find any inositol in the fertilised unincubated hen's egg, but reported that he had been able to isolate a small quantity from it after it had been suspended in saline for some hours. Klein [1909] was also unable to isolate any from the fresh egg, but succeeded in isolating "eine reichliche Menge der Inosit" from eggs which had been allowed to incubate twenty days. His conclusion was that the chick had either made it by synthesis or that it had arisen from some precursor. Lastly, Starkenstein [1911] isolated 20 mg. % from the yolk of a fresh egg. No other researches have been carried out on the cyclose of the egg.

Methods.

In any one experiment the eggs were all of the same breed. They were brought to the laboratory packed in sawdust and after having been aired for half-an-hour, they were placed in electric incubators kept at a temperature of 101° F. and an atmosphere saturated with water vapour. The estimation method was not found to need any great modification, but had to be adapted to the new material in certain ways. The eggs to be estimated were broken into evaporating basins, and carefully examined as to age: if the size of the embryo did not correspond with its calculated age, it was rejected. The

amniotic membrane and fluid were counted as chick, the vitelline membrane (when it could be separated) as yolk, and the shell-membrane as shell, with which it was thrown away.

The embryos were pounded up with clean sand and the different fractions then extracted with acetone in the way described in the first paper of this series. After standing overnight, the tissue was removed by straining through muslin and the acetone distilled off. The aqueous residue was next boiled to coagulate the protein which had escaped precipitation with the acetone, and the mixture was then poured into a large separating funnel. It was diluted to about twice its bulk, so that after an hour or so the protein had settled to the bottom and the oily matter had come to the top. The underlying liquid and the precipitate were drawn off into a beaker and the oil washed once or twice with distilled water. Then the combined aqueous fractions were precipitated with neutral lead acetate and the estimation proceeded with as usual. These were the only modifications necessary.

Experiments to determine the changes in the concentration of cyclose as embryogenesis proceeded.

The first experiment was done on eggs from Black Leghorn hens. At first the white and the yolk were analysed separately, but before long the vitelline membrane becomes too fragile to allow of this, and the partition is then into embryo and remainder. The results of this are recorded in Table I, and a graphic presentation of them given in Fig. 1.

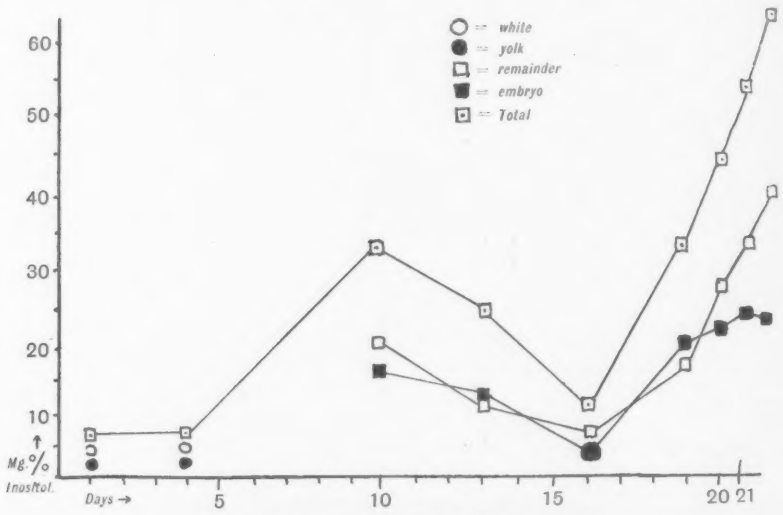

Fig. 1. Inositol-content of *Black* Leghorn eggs.

Table I. *Black Leghorn eggs. Concentration of i-inositol during development.*

Day of Incubation	Fraction	No. of eggs taken	Weight of tissue	cc. of CO_2 per cc. of final solution	Inositol			Total mg. %
					Mg. % of final solution	Mg. % of tissue	Mg. per unit of tissue	
1	White	9	289·5	0·0971	12·6	4·35	1·4	6·792
1	Yolk	9	173·9	0·0351	4·25	2·4	0·47	
4	White and yolk	4	197·2	0·0596	14·24	7·1	2·95	7·221
10	Embryo	12	29·12	0·0338	4·51	15·49	0·37	33·67
10	Remainder	2	93·88	0·1279	17·07	18·18	8·53	
13	Embryo	10	57·7	0·0551	7·35	12·74	0·735	23·97
13	Remainder	1	42·3	0·0356	4·75	11·23	4·75	
16	Embryo	4	63·5	0·0199	2·66	4·19	0·66	12·21
16	Remainder	2	66·8	0·0610	5·20	8·02	3·90	
19	Embryo	2	53·8	0·0722	9·63	17·89	4·81	33·16
19	Remainder	3	53·3	0·0610	8·14	15·27	2·71	
20	Embryo	2	58·0	0·0413	5·51	19·5	2·75	44·84
20	Remainder	3	53·2	0·1009	13·46	25·34	4·48	
21	Embryo	2	70·1	0·1182	15·78	22·54	7·89	51·50
21	Remainder	3	25·0	0·0543	7·24	28·96	2·41	
Hatched	Embryo	2	89·0	0·1416	18·89	21·22	9·44	60·16
„	Remainder	2	16·0	0·0467	6·23	38·94	3·11	

From these data it will be seen that the total inositol in the egg rises from about 7 mg. % at the beginning to over 60 at the end. The general shape of the curve is doubly peaked, the first maximum occurring about the tenth day and the second one being coincident with hatching. Between the two maxima there is a depression in which the total inositol descends to a level not greatly above that which it occupied at the beginning. After the depression, which seems to occur about the sixteenth day, there is a marked rise, and now the amounts in embryo and remainder, which before kept very nearly parallel, begin to differ. The inositol in the remainder increases at the very end of incubation much more rapidly than does that in the embryo.

The significance of the two maxima is a subject which will be returned to later. But attention should be paid at this point to the light which these data throw on the problem of the synthesis of inositol. *A priori* the possibility exists that inositol is contained in the unincubated egg solely in combination with phosphorus in the form of some phytin-like compound. This is, indeed, the supposition which would follow naturally from the general view of Starkenstein [1911]. During development the phytin would break down and free inositol would accumulate. We possess, however, some very reliable figures for the phosphorus-partition of eggs at all stages of their development though, unfortunately, the breed of hen is not given [Plimmer and Scott, 1909], and by their aid we can calculate what amount of water-soluble organic phosphorus would have to be present at the beginning in order to account for all the inositol set free during development. In every molecule of phytic acid there are six atoms of phosphorus: giving a molecular weight of 813 for the phytic acid molecule. It thus appears that the 60 mg. % of free inositol present at the end of incubation would require 62 mg. % of water-soluble

organic P at the beginning, whereas Plimmer and Scott found that the total water-soluble phosphorus in the fertilised but unincubated egg amounted only to 26·64 mg. % P. It is, therefore, clear that if the inositol produced at the end arose from phytin present at the beginning: about three times as much organic water-soluble phosphorus as is actually present would be needed to account for it. Of course the precursor might be a di- or tri-phosphate of inositol; but there is no reason at present to suppose that such compounds occur in the animal body.

Before proceeding to the next experiment, it is interesting to note that the findings of Rosenberger and Klein that no inositol could be found in the undeveloped egg, could not be confirmed. On the contrary, the single figure of Starkenstein is seen to be nearer the real state of affairs.

The second series of experiments was done on White Leghorn eggs, of genuine ancestry, and was in all respects comparable with the first. The results which were obtained are given in Table II, and plotted in the form of a curve in Fig. 2.

Table II. *White Leghorn eggs. Concentration of i-inositol during development.*

Day of incubation	Fraction	No. of eggs taken	Weight of tissue	cc. of CO_2 per cc. of final solution	Inositol			Total mg. %
					Mg. % of final solution	Mg. % of tissue	Mg. per unit of tissue	
0	Yolk	8	171·0	0·3872	51·66	30·21	6·45	33·633
0	White	8	252·75	0·0648	8·65	3·42	1·08	
4	Yolk	3	86·0	0·1728	23·05	26·8	7·68	33·80
4	White	3	59·0	—	—	7·0?	2·50	
6	Embryo	21	23·0	0·4342	57·94	252·0	2·76	302·06
6	Remainder	3	149·0	0·5590	74·59	50·06	24·86	
7	Embryo	14	28·0	0·4566	60·94	217·60	—	250·00?
9	Embryo	8	40·0	0·3982	53·14	132·80	6·64	160·8
9	Remainder	5	250·0	0·5400	70·00	28·00	14·0	
12	Embryo	6	50·0	0·2116	28·33	56·46	4·7	74·12
12	Remainder	4	250·0	0·3808	44·14	17·66	11·03	
15	Embryo	2	60·0	0·4980	66·45	110·80	33·22	257·2
15	Remainder	2	55·0	0·6036	80·54	146·40	40·27	
18	Embryo	3	74·0	0·6968	92·98	125·50	30·99	393·7
18	Remainder	3	57·0	1·1454	152·80	268·00	101·86	
21	Embryo	3	60·0	0·8400	112·1	186·8	37·30	450·0?

In the shape of the curves a striking resemblance is seen. There are two maxima as before; the only difference is that the time-relations are slightly different. The same relations between embryo and remainder at the end of development hold, however, in the second breed, for the inositol in the remainder increases much more rapidly than it does in the embryo in the final stages of incubation. But quite the most remarkable point about the two sets of experiments is that in the White Leghorn eggs, the quantities concerned are two or three times as great as in the Blacks, while yet the shape of the curve remains the same. Thus, the inositol in the whole Black Leghorn egg is 33 mg. % on the tenth day, in the White it is 74 mg. % on the twelfth day. Other points on the curve exhibit even greater difference. Accordingly the

probability that the inositol is not formed from phytin becomes even greater.

This state of affairs is very difficult to account for. That the breed of hen is a highly important matter in dealing with developmental work, is shown by the analyses of Langworthy [1906] and the experiments of Moran [1924]. The former, in an extended series of researches on the chemical constitution of eggs, found considerable differences in their composition. Moran later found that eggs varied greatly in their resistance to cold, and the temperature of their optimum viability.

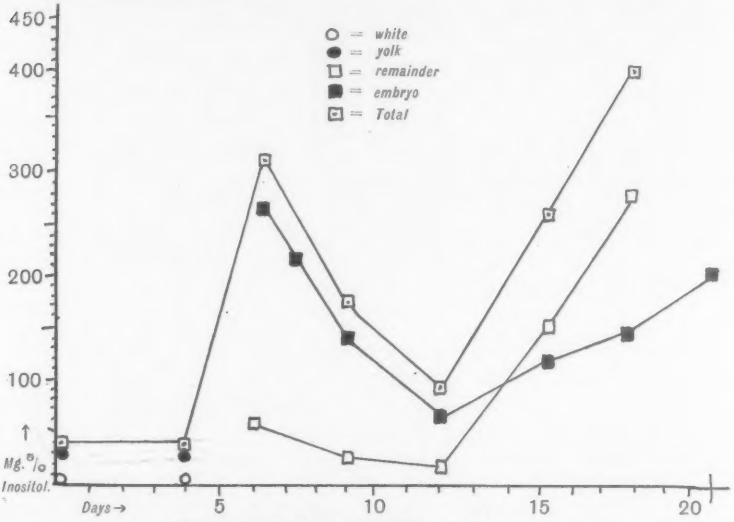

Fig. 2. Inositol-content of *White* Leghorn eggs.

The fact that the difference here is between a coloured and an uncoloured breed brings up at once the possibility that the phenomenon may have something to do with that factor. Unfortunately there is no indication anywhere in our knowledge of inositol that it might have some part to play in this connection. White Leghorns are a breed in which the white character is dominant, and the writer is proposing to repeat these estimations on eggs from White Minorca hens, in which the colour factor is recessive.

Experiments to determine the precursor of the cyclose of the egg.

From the results which have already been described in this paper, it seems certain that whatever may be the source of the *i*-inositol in the egg, that source cannot be phytin. In order to gain further information as to what its precursor might be, it was decided to make use of the injection-method devised by Tomita [1921], who injected glucose into hen's eggs and found by

subsequent analyses that the lactic acid content had risen considerably above the controls. This did not occur when he injected alanine. The following experiment was therefore done.

Two hundred eggs of known White Leghorn breed were divided into five equal groups. One group was injected with phytic acid prepared from commercial phytin by the method of Plimmer and Page [1913], each egg receiving 200 mg. The second group was injected with the same quantity of hexamethylenetetramine. The third and fourth groups were injected with glucose, each egg receiving in the first case 250 mg. of glucose and in the second case 125 mg. These groups will be referred to hereafter as Groups P, B, G, and D. The fifth group, C, was used as a control. Hexamethylenetetramine was injected to test the hypothesis of Posternak [1900], who suggested that inositol might arise in the body by the condensation of six molecules of formaldehyde. That hexamethylene does indeed break down into formaldehyde when introduced into the egg was proved as follows. One of the unincubated eggs which had been injected with hexamethylenetetramine was broken into a distilling flask, diluted to 600 cc. with distilled water, and the mixture steam distilled for four hours. The distillate was then tested for formaldehyde and found to contain it in a concentration certainly exceeding 1 in 50,000. On the solution of hexamethylenetetramine that had been injected, the tests were also positive but exceedingly feeble.

The injection of the eggs was accomplished in the following manner. The pointed end of each egg was painted over with an alcoholic solution of iodine, and a small round hole drilled in the shell with a dentist's burr. The liquid to be injected, having previously been sterilised, was then introduced into the egg with the aid of a sterile hypodermic syringe, and the albumin which exuded wiped off. Finally a small square piece of adhesive plaster was stuck over the hole, and the egg placed in the electric incubator to develop. The whole technique was done under precautions to ensure sterility, the drill, for example, being well boiled first. It is noteworthy that Tomita, using this method, was unable to get his chicks to develop for more than three days, but in the present experiments, injected eggs developed as far as the fifteenth day. The mortality is always greater among injected than among control eggs, but it is likely that with care in sterilisation and an avoidance of shocks during the drilling, this could be still more reduced, and development proceed as far as hatching.

The results obtained in this way are recorded in Table III and Fig. 3. The figures were not all obtained in the first experiment; they are the summarised results of all. The eggs of Group P and Group H did not develop at all. On the seventh day the eggs of Group P possessed an unpleasant smell, and showed traces of decomposition. The white was coloured brown and there were distinct evidences of coagulation around the point of injection. In some cases there had been development for one or two days. The eggs of Group H were also unfertile. The yolk was seen to be wrinkled in appearance by the seventh day, and this wrinkling became progressively more pronounced. It was accom-

panied by an increasing viscosity of the yolk, so it was clear that the normal current of water yolkwards [Riddle, 1916; Aggazzotti, 1923] had been reversed. There was, however, no sign of decomposition or coagulation. Groups G and D developed excellently.

Table III. *Effect of injection of glucose.*

Day of incubation	Description: Series	Description: Fraction	No. of eggs taken	Weight of tissue	cc. of CO_2 per cc. of final solution	Inositol: Mg. % of final solution	Inositol: Mg. % of tissue	Inositol: Mg. per unit of tissue	Total mg. %
7	G	Embryo	17	18·0	0·5854	78·22	434·5	25·57	476·5
7	G	Remainder	4	190·0	0·5986	79·87	42·04	19·97	
7	G	Embryo	6	12·0	0·4973	66·37	553·1	92·17	582·9
7	G	Remainder	3	146·0	0·3261	43·51	29·8	10·0	
7	C	Embryo	14	28·0	0·4566	60·94	217·6	15·54	—
7	D	Embryo	14	25·0	0·5336	71·22	284·9	5·088	313·09
7	D	Remainder	5	240·0	0·5070	67·66	28·19	13·53	
14	D	Embryo	3	40·0	0·5184	69·16	172·9	23·05	260·36
14	D	Remainder	3	100·0	0·6554	87·46	87·46	29·15	
14	H	Infertile whole egg	3	130·0	0·4798	64·01	49·25	21·33	—

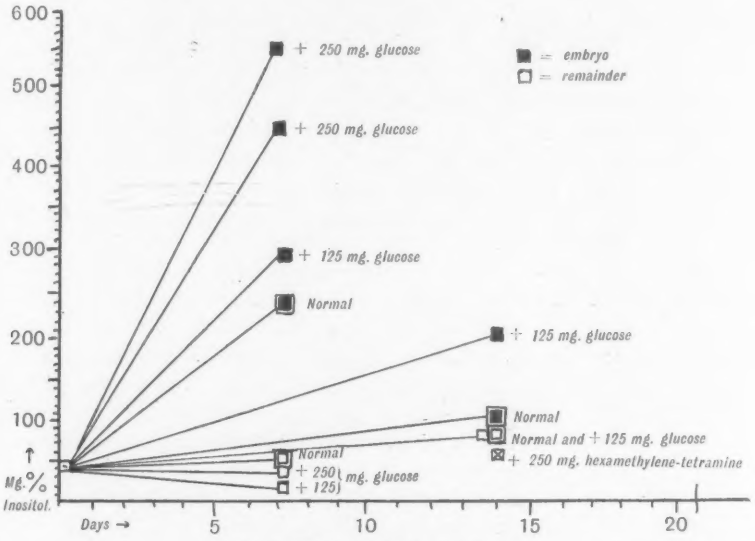

Fig. 3. Effect of injection of glucose.

It is clear from Fig. 3 that the injection of glucose into the egg at the beginning of incubation has a great effect upon the first inositol maximum. In Group D, indeed, this is not very marked at the seventh day, though considerable at the fourteenth. In Group G, on the other hand, in which 250 mg. were injected into each egg, the effect is to double the first inositol maximum,

so that the condition at the seventh day approximates to that found normally at the end of incubation. It is to be noted that in all cases the change is seen only in the embryo. From this fact, it would seem likely that the synthesis of inositol from glucose only takes place in the embryo. This has a bearing on the shape of the curves at the end of incubation, where it was seen that inositol in the remainder rises more rapidly than in the embryo. Perhaps then the inositol is being formed in the embryo more rapidly than the needs of the growing organism demand.

Discussion.

The conclusion that inositol has been synthesised from glucose by the developing embryo can admit of no doubt, but whether this is the normal process, and with what other happenings the shape of the inositol curves should be correlated, is a much more difficult matter. There is evidence, however, that the glucose in the hen's egg sinks continuously from the beginning of development until the tenth day. It must be remembered in the discussion of this subject that no glycogen or other polysaccharide has been found in the avian egg. The free glucose [see Bywaters, 1913; Satô, 1916; Tomita, 1921], which at the beginning of incubation amounts to between 400 and 500 mg. % sinks until at the tenth day there is barely 200 mg. %. The maximum amount of lactic acid formed is about 100 mg. % according to Tomita's figures, and putting the figure for the inositol maximum at 200 mg. %, it is evident that much of the free glucose is accounted for. As a matter of fact, such a calculation fails to take into account the considerable stores of glucose contained in ovomucoid, and it is possible that the second maximum of the inositol may be due to the glucose from that source. Tomita found that a maximum of lactic acid occurred on the fifth day, so perhaps the glucose first forms lactic acid and afterwards inositol. But as to what function the inositol may perform when it has been synthesised we have as yet no clue.

Not a few attempts have been made by earlier workers to demonstrate a relationship between the cycloses and the carbohydrates, a possibility which, from a study of the formula of inositol, had a certain *a priori* likelihood. Greenwald and Weiss [1917] tried to show that glucose was formed when inositol was fed, but their results were not very conclusive, and Anderson [1916] could find no rise in the Respiratory Quotient of dogs after the feeding of inositol. Long previously it had been shown by Külz [1876] and von Mering [1876] that inositol was incapable of producing glycogen in the livers of phloridzinised dogs. Then Oppenheim [1912], Griesbach [1913] and Embden [1914] all obtained entirely negative results when inositol was perfused through various tissues, and glucose or lactic acid looked for. Lactic acid had, indeed, been obtained as a product of bacterial action by Hilger [1871] and Vohl [1876] but, as later workers have shown [Hewitt and Steabben, 1922], it occurs only along with a host of other products, and can have little significance

in this connection. The experiments of Mayer [1907] were also inconclusive, though mainly negative.

All this work failed, then, to relate inositol metabolism to that of the carbohydrates in any way. The noticeable characteristic of it is, however, that all these workers tried to show that inositol could be the precursor of glucose, and none of them that glucose could be the precursor of inositol. But there is no reason why the reaction, glucose $\rightleftharpoons$ inositol, although theoretically reversible, in the body might be so controlled that inositol could be produced from glucose but little or no glucose from inositol. This hypothesis receives support from the effect of insulin convulsions on the free inositol of the rat [Needham, Smith and Winter, 1923]. Insulin convulsions, it was found, raised the free inositol in the tissues of rats by 50 %, although this difference was small compared with the amount of glucose which had disappeared. It seems likely that here also, a change in the carbohydrate side of the equation has resulted in a change on the cyclose side.

In spite of the superficial similarity between the formulae of *i*-inositol and glucose, to picture how the transfer takes place is not easy. That the formation of rings in the body is possible, is of course well known; the case of kynurenic acid need only be referred to. Other cases are also known [see Kikkoji and Neuberg, 1909; Stolte, 1908; Spiro, 1907].

In the last paper of this series [Needham, 1924] it was shown that the body probably possessed the power of synthesising inositol, and in view of the data reported in this paper, it seems probable that it may do so from its supplies of glucose. But it is too early as yet to say so definitely, and there may well be more than one manner in which the cycloses can be produced in animal metabolism.

Summary.

1. The changes in concentration of *i*-inositol during the development of the avian embryo have been studied in detail. Although the absolute quantities concerned differ for Black and White Leghorn eggs, yet the shape of the curve is the same.

2. The injection of glucose into the fertilised unincubated egg causes a very large rise in the inositol content of the egg during its subsequent development. Accordingly it may be considered likely that glucose is the normal precursor of inositol.

The writer's thanks are due to Professor F. G. Hopkins for his unfailing help and inspiration. He also wishes to thank particularly Miss Dorothy Moyle, Miss Margaret Whetham and Mr Douglas Edwards, for their help in the injection-process. Finally he is grateful to Messrs Chivers and Sons for supplying the eggs used in this research.

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CLXXXVI. SOME COLLOIDAL PROPERTIES OF WASSERMANN ANTIGENS.

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A very large amount of work has been done on the more direct and obvious properties of the colloidal lyophobe sols known as "antigens," which are used, for example, in carrying out the Wassermann, Sachs-Georgi or Sigma reactions. So far as we are aware no general investigation of the colloidal properties of these antigens has been made from a fundamental point of view.

Such antigens are prepared by mincing the tissue of an organ, preferably bullock or human heart, the material in a dry or moist state being then, with or without preliminary extraction with acetone, extracted with alcohol under given conditions. On adding the alcoholic extract to normal saline solution an opalescent sol results. This sol added to serum which has been first heated to 55° (inactivated) causes under certain conditions the formation of a precipitate, and under certain other conditions it can be shown that if in addition some fresh serum (complement) is added, the mixture when incubated at 37°, loses its power of haemolysing red blood corpuscles sensitised by specific immune body (the Wassermann reaction). This is hypothetically regarded as being due to some kind of incipient precipitate formation which results in the binding of the hypothetical "complement" present in the fresh serum added and necessary for the haemolysis of the cells. When the complement is "bound" it cannot act in producing haemolysis. It appears to be fairly well established that such precipitation (Sigma test), or fixation of complement (Wassermann test), by antigen plus serum, occurs with a very high percentage of sera from patients suffering from syphilis, and is rare, if it exists at all, in the absence of such syphilitic infection, but the physical or chemical mechanism at the basis of these reactions is very obscure.

It was considered that an investigation of the physical properties of these Wassermann antigens on the lines adopted in the paper on Colloidal "Gum Benzoin" [Wright and Kermack, 1923], would be of much interest. However, it soon became apparent that it was desirable to investigate more closely such questions as the magnitude of the potential difference between the particles of the sol and the medium. We therefore decided to investigate quantitatively the simple case of a colloidal solution of cholesterol, an important constituent of Wassermann "antigens." The results of this investigation will be described in a later paper. Meanwhile the experimental results obtained with the Wassermann "antigens" themselves are here recorded.

A reference may be made to the very interesting results obtained by Holker [1922] on the effect of increasing concentrations of sodium chloride on the antigen. Using the antigen of Bordet and Ruelens (cholesterolised), with progressive variation of sodium chloride concentration, he found rhythmic alternations in the opacity of the antigen, an effect particularly obvious where his special opacimeter is used, but also visible to the naked eye. We have been concerned rather with the gross alteration in dispersion of the nature of total precipitation. It is of interest to note that even very great concentrations of sodium chloride do not cause the complete precipitation which is effected quite easily by calcium chloride.

I. Preparation of Antigen.

The method of preparation of the "antigen," is that described in the Medical Research Committee's Report on the Wassermann test [1918]. It is a modification of that of McIntosh and Fildes [1916]. The muscle tissue of human heart, obtained as fresh as possible and freed carefully from fat with scissors, was minced finely in a mincing machine. The material was weighed, placed in a Winchester bottle with a closely fitting stopper, and for each gram 10 cc. of absolute alcohol were added. The bottle was stoppered, and sealed over with plasticine, and, after its contents had been thoroughly shaken, was placed in an incubator at 37° for 21 days. The mixture was well shaken up daily, and at the end of the period was filtered, a clear colourless fluid being obtained. This extract, without the addition of cholesterol, was the antigen, McIntosh and Fildes I. The antigen McIntosh and Fildes II was prepared in a similar manner from the muscle tissue of bullock's heart.

In addition to the above antigen (McIntosh and Fildes I) which was the one used in general, two other antigens were also prepared and used for comparison. Their modes of preparation are briefly as follows:

Antigen of Keim and Wile [1922] was prepared by drying by means of a fan a thinly spread layer of finely minced fresh bullock's heart muscle tissue and grinding up the dried product. This was extracted for 24 hours in the ice chest with fresh ether on four successive days. After filtration the tissue was dried at room temperature till odourless, and extracted with 100 cc. of absolute alcohol for each 20 g. for nine days in the ice chest and one day at room temperature. The alcoholic filtrate (cholesterolised) was used in preparing the antigen.

Antigen of Bordet and Ruelens [1919] was prepared by first extracting 100 g. of finely minced calf's heart muscle tissue with 125 cc. of 95 % alcohol for five days at room temperature. The residue from filtration was spread in a thin layer and dried at 37° till dark in colour and just brittle, and then extracted with 200 cc. of pure acetone for ten days at 21°, shaking daily. The acetone was poured off and replaced with 100 cc. of fresh acetone for six hours. The residue from filtration was dried for two hours, and after grinding to a coarse

powder, was extracted for 20 days (with daily shakings) at 21° with 200 cc. of 95 % alcohol. The filtrate (non-cholesterolised) was used in preparing the antigen.

For use, 10 cc. of the antigen of McIntosh and Fildes was placed in the bottom of a dry 100 cc. measuring cylinder, and 90 cc. of distilled water were poured in rapidly and the mixture shaken vigorously. The antigen thus prepared differs from that used in the Wassermann reaction in that it contains no sodium chloride. It was considered that this variation was advisable as sodium chloride could be added as required and it seemed important to investigate the antigen first of all in the absence of that salt. In the experiments described below Keim and Wile's extract was similarly treated. The antigen of Bordet and Ruelens, however, was prepared by taking 1 cc. of the extract and evaporating to dryness in a porcelain basin. The yellowish residue was dispersed in 2 cc. of distilled water by triturating the material with a glass rod and the result was diluted to 10 cc. with distilled water. The determination of the hydrogen ion concentration in the following experiments was carried out colorimetrically with the Clark-Lubs sulphonephthalein series of indicators [Clark, 1920].

II. Precipitation by Acids and Salts.

(*a*) *Precipitation by Acid.*

Compared with ordinary suspensoid sols, a very high concentration of acid is required to precipitate Wassermann antigens. A comparison of the action of various acids has been made on four antigens. The results are shown in the following table:

Table I. *Effect of Acids on Antigens.*

Antigen	Concentrations of sulphuric acid					Concentrations of hydrochloric acid				
	N/20	*N*/40	*N*/80	*N*/160	*N*/320	*N*/20	*N*/40	*N*/80	*N*/160	*N*/320
Keim and Wile (1 in 10)	4 (p_H 1·6)	4 (p_H 1·8)	1 (p_H 2·2)	0	0	4 (p_H 1·6)	2 (p_H 1·9)	0 (p_H 2·4)	0	0
McIntosh and Fildes I (1 in 10)	3 (p_H 1·6)	3 (p_H 1·8)	1 (p_H 2·0)	0	0	4 (p_H 1·9)	3 (p_H 2·1)	2 (p_H 2·4)	0 (p_H 2·8)	0
McIntosh and Fildes II (1 in 10)	4 (p_H 1·4)	2 (p_H 1·6)	1 (p_H 2·0)	0	0	4 (p_H 1·6)	2 (p_H 1·9)	0 (p_H 2·4)	0	0
Bordet (1 in 10)	4 (p_H 1·4)	2 (p_H 1·6)	0 (p_H 2·0)	0	0	op. (p_H 1·6)	0 (p_H 2·1)	0	0	0
Bordet (1 in 40)	op. (p_H 1·6)	0 (p_H 1·8)	0	0	0					

Antigen	Concentrations of acetic acid				
	N/20	*N*/40	*N*/80	*N*/160	*N*/320
Keim and Wile (1 in 10)	4 (p_H 1·4)	0 (p_H 2·0)	0	0	0
McIntosh and Fildes I (1 in 10)	4 (p_H 1·4)	2 (p_H 2·0)	0 (p_H 2·4)	0	0
McIntosh and Fildes II (1 in 10)	3	3 (p_H 1·8)	0 (p_H 2·3)	0	0
Bordet (1 in 40)	op. (p_H 1·4)	0 (p_H 1·9)	0	0	0

The antigen of Bordet and Ruelens as prepared contains little solid material and an opalescence (op.) rather than a precipitation was obtained. In the other cases 4 denotes complete precipitation, 3 almost complete precipitation, 2 partial precipitation, but supernatant fluid not nearly clear, 1 very slight precipitation, and 0 no precipitation.

It will be seen that, as far as the experiments in the above table go, it is the hydrogen ion concentration which is the determining factor in precipitation by acids. Precipitation is always obtained at p_H 1·8 or 1·9 in the case of the antigens of Keim and Wile or McIntosh and Fildes II (bullock's heart), whilst the human heart antigen (McIntosh and Fildes I) appears to be slightly more difficult to precipitate, requiring a p_H of 1·6, and Bordet's antigen, which is so dilute that it gives only an opalescence, behaves similarly.

(*b*) *Behaviour in the presence of Sodium Chloride.*

No precipitation of antigen (human heart) was obtained on adding sodium chloride in concentrations from 10 % to 0·0045 % with variations in the p_H of from 6·4 to 3·5.

(*c*) *Precipitation by Calcium Chloride.*

It was found that calcium chloride in comparatively low concentrations produced precipitation of the antigen, but that such precipitation did not occur in the presence of larger amounts of the salt. At first sight this appeared rather similar to the zone effect observed, for example, with ferric chloride and colloidal "gum benzoin," but that the phenomena are different would appear from the fact that precipitation did not again occur in the presence even of very great concentration of calcium chloride. It was thought to be of interest to investigate the effect of variation of p_H on this action of calcium chloride on the antigen, and the results are shown in Fig. 1. It will be noticed that the highest concentration of calcium chloride at which precipitation was obtained is 0·625 %, and is little if at all influenced by the hydrogen ion concentration, and in the same way, within wide limits, the minimum amount required to produce precipitation is also independent of the reaction.

III. Precipitation by Gelatin.

In view of the comparatively great stability of the antigen, it was of particular interest to ascertain whether its behaviour towards gelatin was similar to that of normal lyophobic sols such as "gum benzoin" [Wright and Kermack, 1923], or cholesterol. The following experiment shows that it is. The isoelectric gelatin was prepared as described by Loeb [1922], and contained a negligible amount of ash. A series of solutions of decreasing concentration was prepared and also a similar series of dilutions of acid. Test tubes were arranged in rows and columns so that each row corresponded to a certain concentration of gelatin and each column to a certain concentration of acid (or of distilled water). Into each test tube was put 0·5 cc. of the proper dilution of gelatin, 0·5 cc. of the appropriate solution of acid (or distilled water), and 1 cc. of the antigen. The contents of the tubes were thoroughly mixed, the tubes corked, and allowed to stand overnight. The results are shown in Fig. 2. The nature of the charge on the particles determined by observation

of their behaviour in an electric field under the ultramicroscope is also indicated. It will be noted from the figure that the zone of precipitation, as in the case of gum benzoin, separates the positively from the negatively charged region and that this zone does not extend beyond a p_H of 4·7.

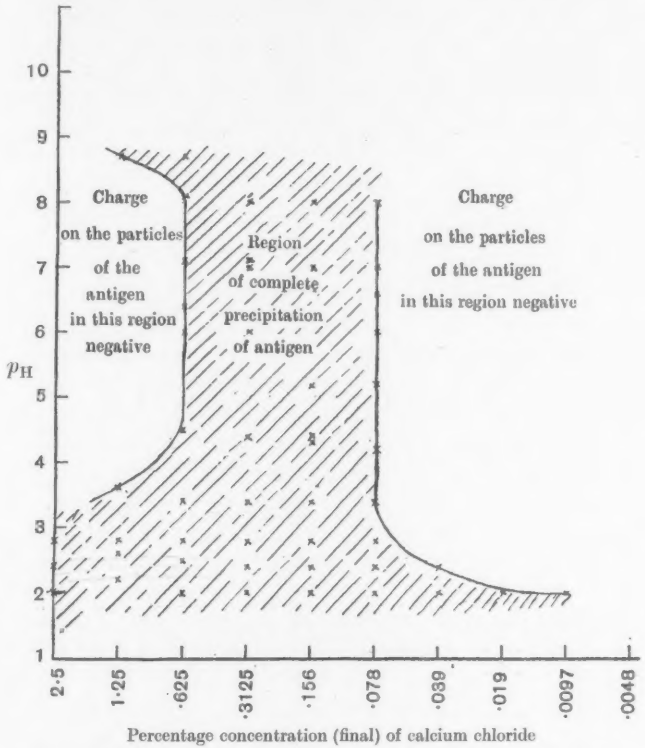

Fig. 1. Effect of p_H on the precipitation of "Antigen" by calcium chloride.

It may be noted that gelatin without the addition of acid does not produce a precipitate with the antigen as it does with the "benzoin," since the resultant p_H without the addition of acid is about 5·0, and therefore on the alkaline side of the isoelectric point of gelatin. All the gelatin exists as negatively charged anion and so does not cause precipitation.

Discussion.

It will be noticed that in several respects a Wassermann antigen of the type investigated differs from an ordinary sol. The differences are particularly obvious in connection with the precipitation by salts. Sodium chloride, which

in comparatively low concentrations precipitates colloidal gold or colloidal "gum benzoin," does not precipitate the antigen even in very high concentration. In the same way calcium chloride does not precipitate the antigen till a concentration of about 0·07 % is reached, and, when a concentration of 1·25 % or more is used there is no precipitation but rather a protective effect. When acid alone is used it will be observed that whereas colloidal gold and colloidal "gum benzoin" are precipitated at about p_H 2·8 or 3·0, the Wassermann antigen is not precipitated till the p_H is as low as 1·9. Notwithstanding the great stability of the antigen, it is of interest to note that the precipitation by protein appears to be as marked as ever and apparently as normal.

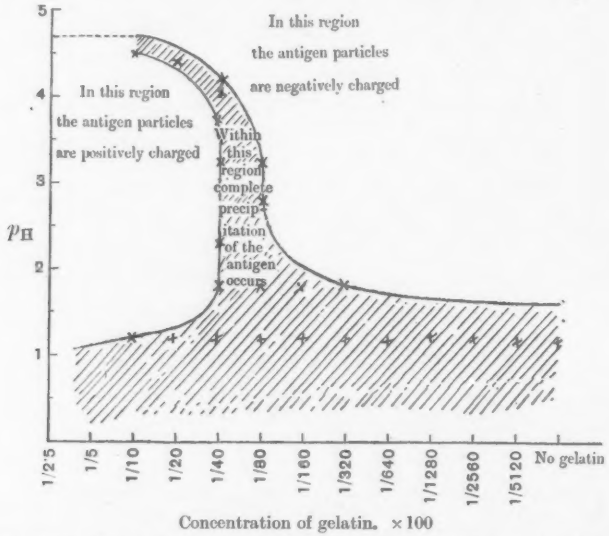

Fig. 2. Illustrating the effect of gelatin on the stability of "Antigen" at various p_H. × indicates actual observations.

It appears probable that the stability of a sol depends on two factors: the particles, aggregates, micelles or whatever units compose the discrete phase may have an electric charge which prevents close contact and coalescence of these units; on the other hand, these units may themselves contain, or they may be associated with, molecules containing groups of atoms whose affinity for water is great enough to overcome the tendency of the particles to aggregate. This tendency to aggregate is of course presumably occasioned by the decrease of surface energy which accompanies such aggregation. Such views have been advanced by Loeb [1923] and confirmatory evidence is given elsewhere.

Precipitation by gelatin is apparently the result of a neutralisation of the electric charge on the particles of the antigen, the gelatin used being too small

in amount to enable its hydrophile groups to resist the tendency of the sol towards aggregation. As noted above, the precipitation of antigen particles by gelatin does take place. The fact that sodium chloride does not effect precipitation would appear then to be due to its being unable to reduce the charge on the particles sufficiently. Measurements of the actual cataphoretic potential difference (P.D.) between the particles and the medium are being made.

The presence on the surface of the particles of molecules of a fairly strong acid which can form an insoluble calcium salt might explain the phenomena observed. Sodium chloride even in high concentrations would not be able to diminish the degree of ionisation of the acid and thus decrease the cataphoretic P.D. between particles and medium caused by the negatively charged ions of the acid, nor, if sufficient acid ions are present, will the Donnan equilibrium effect of sodium chloride (or of monovalent salts in general) on layers of indiffusible ions result in a sufficiently large decrease of the charge. In the same way to obtain precipitation by acid, a relatively high concentration will be required. On the other hand, a small quantity of gelatin, owing to its tendency to be concentrated at interfaces, will be sufficient to neutralise the charge. Also, because of the hypothetical insolubility of the calcium salt of the acid, calcium salts will precipitate the sol in comparatively low concentrations. The exact mechanism of these phenomena can, of course, only be elucidated by further experimental investigation.

Conclusions.

1. An alcoholic extract of heart muscle when added to water forms an opalescent sol which is not precipitated by even high concentrations of sodium chloride, and by acid only when a p_H as low as (approx.) 1·9 is reached. It is precipitated by an equal volume of 0·15 % calcium chloride solution, but not by an equal volume of calcium chloride solution of 2·5 % or more. This action of calcium chloride is little influenced by the p_H.

2. Precipitation of the antigen sol by solutions of gelatin of the requisite (low) concentration occurs at p_H values below 4·7. In this respect the sol resembles the "benzoin" sol previously investigated.

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CLXXXVII. THE CARBOHYDRATE ENZYMES OF SOME STARCH-FREE MONOCOTYLEDONS.

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Investigations on the carbohydrates of plant tissues have hitherto almost neglected one method of attack, namely, the examination of the enzymes present in the plant. If the generally accepted theory of enzyme action be correct, that they catalyse both synthesis and hydrolysis of the higher carbohydrates, then the presence or absence of one or more of the chain of enzymes involved in the series of reactions which result in the formation of starch and other polysaccharides from hexose sugars, may afford valuable evidence why different plants produce different carbohydrates. Especially might this be the case for "starch" and "sugar" leaves.

Starch is hydrolysed to glucose by a series of reactions catalysed by amylase, dextrinase and maltase; the synthesis of starch from glucose presumably requires the same chain of enzymes. If one of them be missing, the synthesis of the full series of carbohydrates, maltose, dextrin and starch, should be impossible, and instead of starch as the end-product some other substance would be formed.

The carbohydrates of plant tissues have only been thoroughly worked out in a few cases, *e.g.* the classical researches of Brown and Morris on the foliage leaves of *Tropaeolum majus* [1893], the work of Parkin [1911] on the green leaf of the snowdrop, and the series of researches by Davis, Daish and Sawyer [1915] on the leaves of the starch-containing potato and the starch-free mangold. Davis and his co-workers strongly criticised the results of Brown and Morris, on the grounds that their methods of preparing the leaves for analysis, by drying in air at super-normal temperatures, would afford opportunity for enzyme action to take place, and state that the maltose which Brown and Morris found present was due to hydrolysis of starch. On the other hand, they consider Parkin's results trustworthy. He found glucose, laevulose and sucrose present in considerable quantity, but no maltose, dextrin or starch, the snowdrop being a typical "sugar" plant. Priestley [1913] had stated that the starch-free snowdrop contained diastase but no maltase, but this was based on only qualitative experiments. It was therefore thought that the green foliage leaves of the snowdrop (*Galanthus nivalis*) would form

an excellent subject for a detailed investigation into the presence or absence of the enzymes concerned in carbohydrate synthesis. Two other typical "sugar" plants, the onion (*Allium cepa*) and the closely-related leek (*Allium porrum*) were also examined, together with a typical "starch" plant, a common dock (*Rumex obtusifolius*), as a control.

Material and Methods.

The method finally elaborated, after various vain efforts to use fresh leaves, was to use dried leaf-powder. The fresh green leaves were usually received by post on the day after collection, and were at once spread out to dry on papers in a well-ventilated room, screened from the direct rays of the sun, which might have had a harmful effect on any enzymes present, especially maltase. When the leaves were quite dry and crisp, they were crumbled by hand and then ground as finely as possible in a mill. The leaf-powder was kept in tightly-corked bottles, screened from light by black paper.

The experiments were conducted in two parallel series, one being a control which had been heated at least 15 minutes in boiling water to destroy all the enzymes present. The usual procedure was as follows: 2·00 g. leaf-powder was put into a clean, dry flask of about 70 cc. capacity, 10 cc. water added and the control put in boiling water; 50 cc. of the substrate and 2 cc. toluene were added, and the flasks incubated at 39° ± 0·5°. The loss of water from the control during the inactivation of the enzymes by heat was prevented as far as possible by leaving the stopper of the flask loosely inserted in the mouth. A test showed an actual loss equivalent to an increase of 0·0035° rotation with a 2 % solution of maltose in a 100 mm. tube. This is about the size of the usual experimental error, and is not significant.

The substrates used were Kahlbaum's soluble starch, dextrin and maltose. The latter ran out in the middle of the work, and was replaced by maltose from Messrs Reynolds and Branson, which gave a pure maltosazone, free from glucosazone, and a rotation corresponding to more than 99 % maltose. Recrystallised sucrose from British Drug Houses and amygdalin prepared by myself were also used.

The tests used were partly qualitative and partly quantitative. The latter was the optical rotation, as measured by a Hilger polarimeter reading to 0·01°. The usual probable error was less than 0·010°, and in no case was it more than 0·038°. The actual tests were the following:

Amylase. Tests with 1 % iodine in potassium iodide for the disappearance of starch and the appearance of dextrin, and phenylosazone tests for the appearance of maltose. 1/24,000 of starch gave a definite coloration with iodine.

Dextrinase. As for starch. 1/10,000 of dextrin gave a coloration with iodine. Polarimetric measurements were also made. Complete hydrolysis of 1 % solution of dextrin to maltose shows a change in rotation from + 1·95° to + 1·45° (*i.e.* − 0·50°); to glucose from + 1·95° to + 0·59° (*i.e.* − 1·36°).

Maltase. The chief test was polarimetric. A 2 % solution on hydrolysis changes from + 2·76° to + 1·11° (*i.e.* − 1·65°). Osazone tests were also made.

Invertase. As for maltase. Complete hydrolysis shows a change from + 1·33° to − 0·42° (*i.e.* − 1·75°).

Emulsin. A qualitative test only was made, using alkaline picrate paper suspended over the solution. 1/50,000 of hydrocyanic acid gave a strong reaction after about 19 hours at 39°.

1 to 2 cc. toluene were used as antiseptic usually, as Daish [1916] showed that this has little effect on maltase, which gives very variable results with ordinary chloroform and alcohol. Chloroform was used sometimes when testing for diastase or emulsin.

(Other methods which were tried before the foregoing technique was finally evolved may be mentioned briefly. All gave disappointing results. Fresh leaves were frozen in liquid air, or solid carbon dioxide and ether, then thoroughly powdered in a mortar. On testing for maltase, none could be found, though experiments with dried leaves of the same plant (dock) gave positive results. Efforts to extract maltase from fresh leaves, after freezing and powdering, also failed. Attempts to release maltase from the protein-protoplasmic complex of the cell by digestion with trypsin and pepsin with hydrocyanic acid as antiseptic also were unsuccessful.)

After incubation (in experiments where the rotation was required) the solutions were filtered, basic lead acetate added to precipitate proteins, etc., the solution again filtered, solid sodium sulphate added to precipitate the slight excess of lead, and after a drop of 0·880 ammonia had been added to bring the solution to equilibrium, the rotation was measured. The solutions were always rather yellow, but this gave no trouble with a sodium flame except in excess, when the polarimeter was adjusted to let more light through with a resulting slight increase in the probable error. The probable error was worked out by the formulae given by Mellor [1922].

Experimental Results.

Column 4 of the tables gives the rotations corrected to a 100 mm. tube, for dilution by lead acetate and to an original solution of 2·00 % for maltose and sucrose (or 1·00 % for dextrin and starch). A rough attempt has been made to indicate the quantity of osazone precipitated by a standard technique, by the terms very much, much, fair, some and trace.

Emulsin (see Table I). The onion, leek and dock showed a strong reaction for hydrocyanic acid after 5 to 21 hours at 39°; the snowdrop failed to show it even after 74 hours. There is therefore no emulsin in the snowdrop, but it is present in all the others.

Invertase (see Table II). The leek and dock showed a decrease in rotation when sucrose was present as compared with that when sucrose was absent, of almost exactly the theoretical amount for complete inversion to glucose and

laevulose; the onion showed rather more, and the snowdrop considerably less decrease, but the results indicate the presence of invertase in all the four leaves. The osazones confirm this.

Table I. *Emulsin.*

0·8 g. leaf + 20 cc. 1 % amygdalin + chloroform.

Expt. no.	Incubated (hours)	Picrate paper turned	Remarks and conclusions
Snowdrop			
X 62	74	Yellow	No emulsin present
Onion			
X 61	21	Red	Emulsin present
Leek			
X 65	5¼	Red	Emulsin present
Dock			
X 60	21	Deep red	Emulsin present

Delicacy of the test for Hydrocyanic acid.

Known strength of solution of HCN	1/1000	1/5000	1/10,000	1/50,000
Incubated for	4 mins.	2½ hours	2½ hours	18¾ hours
Yellow picrate paper turned ...	Pink	Deep pink	Deep pink	Deep red

Table II. *Invertase.*

Exp. no.	Details of experiment (toluene as antiseptic)	Incubated (hours)	Rotation (corrected)	Change	Remarks
Snowdrop					
X 105	Leaf-powder I + cane-sugar	88¼	+0·77° ± 0·002°	−0·71°	No maltosazone; much glucosazone
X 106	Control of *X* 105 (15 mins. at 100°)		+1·48° ± 0·003°		No maltosazone; good glucosazone
X 123	Leaf-powder III + water	68¼	−0·03° ± 0·003°	−0·12°	Trace maltosazone; good glucosazone
X 124	Control of *X* 123 (15 mins. at 100°)		+0·09° ± 0·002°		" "
X 103	Leaf-powder I + water	308¼	−0·16° ± 0·004°	−0·41°	
X 104	Control of *X* 103 (15 mins. at 100°)		+0·25° ± 0·005°		
Onion					
X 109	Leaf-powder I + cane-sugar	64½	−0·72° ± 0·003°	−2·10°	No maltosazone; very heavy glucosazone
X 110	Control of *X* 109 (15 mins. at 100°)		+1·38° ± 0·002°		No maltosazone; good glucosazone
O 13	Leaf-powder III + water	45½	−0·02° ± 0·014°	−0·09°	Trace maltosazone; no glucosazone
O 14	Control of *O* 13 (30 mins. at 100°)		+0·07° ± 0·008°		Trace maltosazone; some glucosazone
O 3	Leaf-powder III + water	234½	−0·14° ± 0·004°	−0·10°	Trace maltosazone; good glucosazone
O 4	Control of *O* 3 (30 mins. at 100°)		−0·04° ± 0·009°		" "
Leek					
X 107	Leaf-powder I + cane-sugar	237¾	−0·33° ± 0·002°	−1·67°	No maltosazone; very heavy glucosazone
X 108	Control of *X* 107 (15 mins. at 100°)		+1·34° ± 0·003°		No maltosazone; some glucosazone
L 3	Leaf-powder II + water	41¼	−0·02° ± 0·006°	−0·10°	No maltosazone; little glucosazone
L 4	Control of *L* 3 (30 mins. at 100°)		+0·08° ± 0·004°		No maltosazone; trace glucosazone
Dock					
X 111	Leaf-powder I + cane-sugar	262¾	−0·40° ± 0·003°	−1·71°	No maltosazone; very heavy glucosazone
X 112	Control of *X* 111 (15 mins. at 100°)		+1·31° ± 0·002°		? Maltosazone; fair glucosazone
X 3	200 g. fresh leaves (without midribs) frozen with solid CO_2 and ether. Powdered, shaken 1 hour with 200 cc. tap-water (without antiseptic). 20 cc. filtrate + cane-sugar	71	−0·28°	−1·73°	(Cleared by shaking with 1·4 g. of animal charcoal for half an hour)
X 3*a*	Control of *X* 3 (20 mins. at 100°)		+1·45°		(Cleared similarly)
X 21	Leaf-powder I + water	119	±0·00°	−0·05°	A little glucosazone
X 22	Control of *X* 21 (20 mins. at 100°)		+0·05°		" "

Table III. *Maltase.*

Exp. no.	Details of experiment (toluene as antiseptic)	Incubated (hours)	Rotation (corrected)	Change	Remarks
Snowdrop					
X 39*a*	Leaf-powder I+maltose	90	+2·77°	−0·11°	Much maltosazone; no glucosazone
X 40*a*	Control of *X* 39*a* (20 mins. at 100°)		+2·88°		" " "
X 121	Leaf-powder III+maltose	68¼	+2·98°±0·006°	−0·11°	Good maltosazone; some glucosazone
X 122	Control of *X* 121 (15 mins. at 100°)		+3·09°±0·004°		" " "
S 1	Leaf-powder IV+maltose	70½	+2·63°±0·023°	−0·35°	Much maltosazone; no glucosazone
S 2	Control of *S* 1 (33 mins. at 100°)		+2·98°±0·012°		" " "
S 5	Leaf-powder IV+maltose	141¼	+2·54°±0·010°	−0·42°	Some maltosazone; no glucosazone
S 6	Control of *S* 5 (33 mins. at 100°)		+2·96°±0·019°		Some maltosazone; a little glucosazone
S 11	Leaf-powder IV+maltose	70¾	+2·96°±0·015°	−0·06°	Good maltosazone; some glucosazone
S 12	Control of *S* 11 (30 mins. at 100°)		+3·02°±0·008°		Fair maltosazone; a little glucosazone
X 101	Leaf-powder I+maltose	332	+2·29°±0·004°	−0·37°	
X 102	Control of *X* 101 (15 mins. at 100°)		+2·66°±0·003°		
X 123	Leaf-powder III+water	68¼	−0·03°±0·003°	−0·12°	Trace maltosazone; good glucosazone
X 124	Control of *X* 123 (15 mins. at 100°)		+0·09°±0·002°		Little maltosazone; good glucosazone
S 9	Leaf-powder IV+water	120	+0·19°±0·023°	−0·46°	Trace maltosazone; fair glucosazone
S 10	Control of *S* 9 (30 mins. at 100°)		+0·65°±0·014°		Trace maltosazone; some glucosazone
X 103	Leaf-powder I+water	332	−0·16°±0·004°	−0·41°	
X 104	Control of *X* 103 (15 mins. at 100°)		+0·25°±0·005°		
S 7	*No leaf*; 10 cc. water+50 cc. 2·00 % maltose+1 cc. toluene	141¼	+2·58°±0·013°	nil	Much maltosazone; no glucosazone
S 8	Control of *S* 7 (33 mins. at 100°)		+2·58°±0·006°		" " "
Onion					
X 39	Leaf-powder I+maltose	190½	+1·16°	−1·37°	Maltosazone; glucosazone
X 40	Control of *X* 39 (15 mins. at 100°)		+2·53°		Maltosazone; no glucosazone
O 3	Leaf-powder III+water	234½	−0·14°±0·004°	−0·10°	Trace maltosazone; good glucosazone
O 4	Control of *O* 3 (30 mins. at 100°)		−0·04°±0·009°		" " " "
Leek					
X 70	Leaf-powder I+maltose	209	+1·34°	−0·57°	Maltosazone; some glucosazone
X 71	Control of *X* 70 (18 mins. at 100°)		+1·91°		Maltosazone; no glucosazone
L 3	Leaf-powder II+water	41¼	−0·02°±0·006°	−0·10°	No maltosazone; little glucosazone
L 4	Control of *L* 3 (30 mins. at 100°)		+0·08°±0·004°		No maltosazone; trace glucosazone
Dock					
X 17	Leaf-powder I+maltose	240	±0·00°	−1·73°	? Maltosazone; heavy glucosazone
X 18	Control of *X* 17 (20 mins. at 100°)		+1·73°		Maltosazone; no glucosazone
X 19	Leaf-powder I+maltose	119	+2·15°	−0·68°	No maltosazone; glucosazone
X 20	Control of *X* 19 (60 mins. at 100°)		+2·83°		Maltosazone; no glucosazone
X 21	Leaf-powder I+water	119	±0·00°	−0·65°	A little glucosazone
X 22	Control of *X* 21 (20 mins. at 100°)		+0·65°		" "

Maltase (see Table III). Three different annual crops of snowdrop leaves agreed in showing no difference in the decrease of rotation with maltose present or absent, that is, there is no maltase in the snowdrop. The onion, leek and dock all showed considerably more decrease when maltose was present than when it was absent, *i.e.* these leaves all contain maltase. The osazones confirmed these results.

Dextrinase (see Table IV). In the snowdrop, leek and dock, the dextrin was hydrolysed, *i.e.* dextrinase is present. In the onion, qualitative and quantitative tests failed to show any hydrolysis, *i.e.* there is no dextrinase in the onion.

Amylase (see Table V). The starch was hydrolysed by all the leaf-powders except the leek, *i.e.* the snowdrop, onion and dock all contain amylase, but it is absent from the leek.

Table IV. *Dextrinase.*

Exp. no.	Details of experiment (chloroform as antiseptic, unless otherwise stated)	Incubated (hours)	Rotation (corrected)	Change	Remarks
Snowdrop					
X 28	Leaf-powder I+dextrin+toluene Then added an equal volume of 2 % dextrin, and replaced in the incubator. After a further	15	—	—	Tested with iodine. No colour
		7	—	—	Repeated iodine test. No colour
X 34	Leaf-powder I shaken 1 hour with water+chloroform. Filtered. Made up to 1 % dextrin	47½	—	—	With iodine test. No colour
Onion					
X 36	Leaf-powder I extracted ½ hour with water+chloroform. Made up to 1 % dextrin	21	—	—	With iodine. Brown
		40	—	—	,, ,,
		91	—	—	,, ,,
O 5	Leaf-powder III+dextrin+toluene	¼	—	—	With iodine. *O* 5 Brown. *O* 6 Brown
O 6	Control of *O* 5 (30 mins. at 100°)	20	—	—	,, ,, ,,
		44½	—	—	,, ,, ,,
		65½	—	—	,, ,, ,,
		97¾	—	—	,, ,, ,,
O 1	Leaf-powder III+dextrin+toluene	112¼	+0·96°±0·005°	−0·18°	
O 2	Control of *O* 1 (30 mins. at 100°)		+1·14°±0·004°		
O 13	Leaf-powder III+water+toluene	45½	−0·02°±0·014°	−0·09°	No glucosazone; some maltosazone. With iodine. Brown.
O 14	Control of *O* 13 (30 mins. at 100°)		+0·07°±0·009°		Trace glucosazone; fair maltosazone. With iodine. Brown
O 3	Leaf-powder III+water+toluene	234½	−0·14°±0·004°	−0·10°	With iodine. Brown
O 4	Control of *O* 3 (30 mins. at 100°)		−0·04°±0·009°		,, ,,
Leek					
X 64	Leaf-powder I +water+chloroform. Shaken 1 hour. Extract made up to 1 % dextrin	112½	—		With iodine. Colourless
X 68	Leaf-powder I shaken with 200 cc. water+chloroform. Filtered. Made up to 50 % alcohol. Precipitate dissolved in 70 cc. water. Made up to 0·50 % dextrin	18½		—	With iodine. Very pale brown
		41½		—	? Brown
		73¾	—	—	Colourless
X 117	Leaf-powder II+dextrin+toluene	336	+0·90°±0·004°	−0·25°	With iodine. Colourless } Both much maltosazone and glucosazone
X 118	Control of *X* 117 (15 mins. at 100°)		+1·15°±0·005°		With iodine. Brown }
X 119	Leaf-powder II +0·5 g. $CaCO_3$+dextrin+toluene	336	+0·77°±0·004°	−0·35°	With iodine. Colourless } Both maltosazone and glucosazone
X 120	Control of *X* 119 (15 mins. at 100°)		+1·12°±0·005°		With iodine. Brown }
L 3	Leaf-powder III+water+toluene	41¼	−0·02°±0·006°	−0·10°	With iodine. Colourless } Both glucosazone. Trace of maltosazone
L 4	Control of *L* 3 (30 mins. at 100°)		+0·08°±0·004°		With iodine. Brown }
Dock					
D	Leaves II shaken up with water+chloroform for ½ hour. Extract made up to 0·5 % dextrin	70	—	—	With iodine. Colourless
F	Leaves III frozen with CO_2 and ether. Powdered. Shaken 1 hour with tap-water. Made up to 0·5 % dextrin+toluene. Added more dextrin. Incubated a further	24	—	—	With iodine. Colourless
		22	—	—	,, ,,
X 6	Leaf III shaken 1 hour with tap-water+chloroform. Extract made up to 0·5 % dextrin+toluene	20½	—	—	With iodine. Very pale brown
		70	—	—	,, Colourless

Table V. *Amylase.*

Exp. no.	Details of experiment (chloroform or toluene as antiseptic)	Incubated (hours)	Rotation (corrected)	Change	Remarks
Snowdrop					
X 27	Leaf-powder I+starch	15	—	—	With iodine. Colourless
X 33	Leaf-powder I+water+chloroform. Shaken ½ hour. Extract made up to 1 % starch	47	—	—	With iodine. Colourless
S 19	Leaf-powder IV+starch	72	+0·83°±0·016°	−0·64°	With iodine. Colourless. Maltosazone, trace glucosazone
S 20	Control of *S* 19 (40 mins. at 100°)		+1·47°±0·038°		With iodine. Blue. No maltosazone, trace glucosazone
S 21	Leaf-powder IV+starch	1433	+0·51°±0·019°	−1·05°	With iodine. Colourless
S 22	Control of *S* 21 (40 mins. at 100°)		+1·56°±0·027°		,, Blue
S 9	Leaf-powder IV+water	120	+0·19°±0·023°	−0·46°	Some maltosazone; fair glucosazone
S 10	Control of *S* 9 (30 mins. at 100°)		+0·65°±0·014°		Trace maltosazone; some glucosazone
X 103	Leaf-powder I+water	332	−0·16°±0·004°	−0·41°	
X 104	Control of *X* 103 (15 mins. at 100°)		+0·25°±0·005°		
Onion					
X 35	Leaf-powder I +water+chloroform. Shaken ½ hour. Extract made up to 1 % starch	21	—	—	With iodine. Blue
		40	—	—	,, Purple
		91	—	—	,, Brown
		624	—	—	,, ,,
O 11	Leaf-powder III+starch	45½	+0·70°±0·016°	—	With iodine. Brown
O 12	Control of *O* 11 (30 mins. at 100°)		—		,, Blue. (Could not measure rotation, because solution would not filter.)
O 3	Leaf-powder III+water	234¼	−0·14°±0·004°	−0·10°	
O 4	Control of *O* 3 (30 mins. at 100°)		−0·04°±0·009°		
O 9	Leaf-powder III+starch	1362	+1·25°±0·010°	−0·15°	With iodine. Brown) No maltosazone
O 10	Control of *O* 9 (30 mins. at 100°)		+1·40°±0·012°		,, Blue) much glucosazone
Leek					
X 63	Leaf-powder I +water+chloroform. Shaken 1 hour. Extract made up to 1 % starch	13	—	—	With iodine. Blue
		47	—	—	,, ,,
		112¼	—	—	,, ,,
X 66	Leaf-powder I +water+chloroform. Shaken 1 hour. Filtered. Precipitated with 50 % alcohol. Precipitate dissolved in water. Made up to 0·5 % starch	18½	—	—	With iodine. Blue
		41½	—	—	,, ,,
		73¾	—	—	,, ,,
		166	—	—	,, ,,
		192½	—	—	,, ,,
		456	—	—	,, ,,
X 67	As *X* 66, but made up to 1·25 % starch	18½	—	—	With iodine. Blue
		192½	—	—	,, ,,
		456	—	—	,, ,,
X 113	Leaf-powder II+starch	573½	+0·91°±0·005°	−0·12°	With iodine. Blue) Maltosazone; much
X 114	Control of *X* 113 (15 mins. at 100°)		+1·03°±0·011°		,, ,,) glucosazone
X 115	Leaf-powder II +starch+0·5 g. $CaCO_3$	598	+0·90°±0·004°	−0·13°	With iodine. Blue. Much glucosazone
X 116	Control of *X* 115 (15 mins. at 100°)		+1·03°±0·023°		,, ,, . Maltosazone, glucosazone
L 7	Leaf-powder III+starch	72	—	—	With iodine. Blue) Could not filter after
L 8	Control of *L* 7 (40 mins. at 100°)		—	—	,, ,,) adding lead acetate
L 9	Leaf-powder III+starch	1410	+0·87°±0·036°	−0·23°	With iodine. Blue. Fair maltosazone, very much glucosazone
L 10	Control of *L* 9 (40 mins. at 100°)		+1·10°±0·010°		With iodine. Blue. Much maltosazone, much glucosazone
L 3	Leaf-powder III+water	41¼	−0·02°±0·006°	−0·10°	Trace maltosazone, little glucosazone
L 4	Control of *L* 3 (30 mins. at 100°)		+0·08°±0·004°		Trace glucosazone
Dock					
A	Fresh leaf I; solidified by liquid air, powdered. Pulp extracted with water+chloroform. Extract made up to 1 % starch	19½	—	—	With iodine. Colourless

Brown and Morris [1893] especially note that in leaves containing much tannin, the action of diastase is inhibited. The leaf-powders were therefore tested for tannin as follows. To a strong infusion of the leaf-powder (prepared by shaking 20 g. for an hour with 100 cc. water) 1 % iron alum was added. There was no trace of precipitate or coloration in any of the leaf-extracts. A further test was made. A freshly-prepared solution of 1 % gelatin in 10 % sodium chloride was added drop by drop to 10 cc. of the leaf-extract, till six drops had been added. There was no trace of precipitate or opalescence in any case. Tannin is therefore absent from all these leaves. (The author is indebted for these tests to Mr F. C. Thompson, of the Department of Leather Industries, Leeds University.)

General Discussion.

It has long been known that certain plants normally do not contain starch in their green, foliage leaves. Arthur Meyer [1885] divided plants into five series according to the amount of starch contained in their leaves, varying from "very rich" to "very little," and specifically stated that five species of *Allium* which he examined contained no starch at all. Böhm [1883] showed that certain leaves normally starch-free can be made to form starch by floating the leaves on sugar solutions. He found that strong solutions were more efficacious than weak ones, and that on 20 % cane sugar, starch was formed in the leaves of *Galanthus nivalis* and other starch-free leaves. These results were confirmed by Schimper [1885] for certain starch-free leaves, but all efforts to form starch in the leaves of the onion failed, and he concluded that the onion had lost the power to form starch. Meyer also obtained starch formation in leaves which were normally starch free (such as *Iris* and *Arum* species), by exposure to strong light in 3 % carbon dioxide, but failed with *Allium* again. Schimper crushed fresh leaves with water and added the solution thus obtained to 1 % starch paste. With the exception of the onion, all had liquefied the starch-paste in 24 hours, including the iris. Parkin [1899] found that the very young leaves of the onion may contain starch, but that this is absent from the older plants. The mature leaves of both the leek and snowdrop do not normally contain starch. Rendle [1889] also states that starch may be found round the vascular bundles of young leaves but rarely in older ones. He tried to induce starch formation by floating the leaves on 20 % glucose, but failed. Parkin[1] tried similar experiments with 20 % sucrose and also failed. Mature leaves of the snowdrop, floated on 20 % sucrose and other sugars also failed to show any starch formation; young leaves also usually failed to show starch formation, but in a few cases a little was found near the vascular bundles. That is, the mature leaves which were used in these experi-

[1] On p. 53 of Parkin's paper it is stated that onion leaves were floated on "2 % cane sugar." This is apparently a misprint for "20 % cane sugar," as on p. 52 he states that solutions of "from 5 % to 20 %" were employed

ments would not contain starch, and therefore should not contain the full series of enzymes needed for starch formation. Schimper's experiments with starch paste showed that the onion had no diastase, which agrees with the results of Table IV—that dextrinase is lacking in the onion.

It has been known since at least 1862 that the guard-cells of the stomata contain starch grains which are retained with great tenacity even when the leaf is starved by being kept in darkness. Parkin [1899] found starch in the guard-cells of all the plants he examined, including many starch-free monocotyledons, except species of *Allium*. He correlated the presence of starch with the presence of chloroplasts, and showed that eight species of *Allium* which contain no starch in the guard-cells also have no chloroplasts or any sign of green coloration there: all other plants examined had both starch and chloroplasts in the guard-cells. Normally, 24 hours in darkness is sufficient to remove nearly all the starch from parenchyma cells, and all traces have gone in 48 hours, but Parkin showed that detached leaves of *Narcissus poeticus* retained starch in their guard-cells after seven days, and that even after fifteen days, though the leaves were turning yellow and withering the guard-cell starch had not wholly disappeared. This starch need not be the product of photosynthesis. Plants of *Galanthus nivalis* grown in the dark showed a little starch in the guard-cells of the upper mature part of the leaf, but none in the lower part. Parkin therefore considers that the presence of stomatal starch is to be correlated with the development of the stomata, and that it only appears when these are fully formed and functional.

The work of Böhm, Schimper, Rendle and Parkin in feeding experiments, showing that the mature green leaves of the onion, leek and snowdrop do not form starch when floated on strong sugar solutions, is quite in accordance with the results of Tables III, IV and V, in which it is shown that the complete series of enzymes required for starch formation is not present in any of these leaves.

Schimper's [1885] results seem to indicate that in order to form starch in the leaf, a certain minimum concentration of sugar is necessary. Where this minimum concentration is not normally attained, the plant does not form starch. In certain cases, the formation of starch can be induced by floating the leaf on strong sugar solutions; this will provide the necessary concentration. But in other cases this method failed, *e.g.* the onion, leek and snowdrop, and in these cases the mechanism for the production of starch is lacking, so that the presence of the raw material, sugar, makes no difference.

The guard-cell starch of those plants which do not contain any other starch may be regarded as formed by a mechanism which later disappears. Table III shows that the snowdrop has no maltase. But starch cannot be formed from glucose without maltase (if enzymes are true catalysts), so that presumably in the young leaf, the whole apparatus—maltase, dextrinase and amylase—required to convert glucose into starch is present in the guard-cells. Starch

is formed, and afterwards the maltase disappears. Thus no more starch can be formed, but equally the starch already formed cannot be hydrolysed further than maltose. The series of changes

$$\text{Starch} \rightleftarrows \text{Maltose}$$

under the influence of amylase and dextrinase would form an ideal mechanism for controlling the opening of the stomata if the reaction is

$$\text{Starch} \rightarrow \text{Maltose}$$

when the plant has plenty of water and transpiration is not too intense, and

$$\text{Starch} \leftarrow \text{Maltose}$$

when transpiration is high and water supplies are failing. But these are exactly the circumstances required, because the full equation is

$$\text{Starch} + \text{Water} \rightleftarrows \text{Maltose},$$

and a high water content in the leaf would cause the reaction to go to the right. The maltose thus formed would increase the soluble contents of the guard-cells, water would be taken in by osmosis until turgidity was reached, and the stomata would open. If water were lacking, the reaction would go to the left, maltose would be replaced by insoluble starch, the soluble contents of the guard-cells would be lessened, water would be lost by osmosis, the guard-cells would no longer be turgid and the stomata would close. This system of amylase and dextrinase with no maltase might be described as a "chemical appendix," a relic of an earlier state, but in this case the appendix is still functional and useful. Tables IV and V seem to indicate that there is only a small quantity of diastase in the snowdrop, which would be quite compatible with this theory.

Similarly, the onion and the leek cannot form starch in the green leaf, because the complete starch-forming machinery is again lacking; the onion lacks dextrinase and the leek lacks amylase.

The dock can form starch because it contains the full equipment of maltase, dextrinase and amylase.

Brown and Morris determined the "diastatic activity" of a number of plants, defining it as the number of grams of maltose formed from soluble starch by 10 g. of leaf at 30°. These varied from 240·3 for *Pisum sativum* to 0·267 for *Hydrocharis morsus-ranae*. Three members of the Solanaceae, the potato, tobacco and tomato, which were specially mentioned by Arthur Meyer [1885] as forming abundant starch, have values from 6·57 to 8·16, little removed from that of the onion, 3·76. Brown and Morris do not comment at all on this rather curious juxtaposition and do not say that the Solanaceae have any tannin (which might have inhibited the action of the diastase).

It is interesting to note that in the snowdrop, the β-glucoside-splitting enzyme, emulsin, is also absent, as well as the α-glucoside-splitting enzyme, maltase.

The theory stated above, that where starch is normally found, the full equipment of enzymes, maltase, dextrinase and amylase, should be present,

is confirmed by the results of Mason [1916]. He used essentially the same method as was used for the experiments described in this paper, except that his leaves were treated with 95 % alcohol to remove sugars before being dried and powdered, and instead of measuring the rotations of his solutions, he took the copper-reducing power. He found in *Polytrichum commune* glucose, laevulose, sucrose, a little maltose and starch; he also found diastase and maltase present. In *Thuidium tamariscinum* and *Sphagnum cymbifolium*, he found present glucose, laevulose and sucrose, but no maltose or starch; he also found no diastase in these mosses. That is, diastase and maltase were only found in starch-containing leaves.

Robertson, Irving and Dobson [1909] working on the sugar-beet, *Beta vulgaris*, found present in the leaf and root both diastase and maltase, but in the stem only diastase without any maltase. The leaf and root do not contain starch (except in the very young leaf), though in the leaf, at any rate, there appears to be the full enzyme mechanism and the raw material (glucose) for the manufacture of starch. No mention is made of tannin, which might inhibit the action of diastase *in vivo* as it does *in vitro*, and there is no discussion of the reason for the absence of starch from the leaf, though the authors agree with the view that the same enzyme or enzymes should catalyse both in the direction of synthesis or hydrolysis, according to the conditions present. A variety of the same species, the mangold, was investigated by Davis, Daish and Sawyer [1915]. Starch was found in the very young leaf, when the root was small and not available as a storage organ, but as soon as the root becomes sufficiently large to serve as a storage organ, starch disappears from the leaf. Carbohydrates are stored in the root chiefly as sucrose. Daish [1916] showed that maltase was present in a number of starch-forming plants, *Tropaeolum*, potato, dahlia, turnip, mangold and sunflower, although hitherto maltase had been rarely described as present in foliage leaves. Davis [1915] concluded from these results that maltase would be universally found present in starch-forming plants, and that failure to demonstrate its presence in the past has been due to difficulties of extraction, and to its instability. (It is easily destroyed by temperatures above 50°, and by contact with ordinary alcohol and chloroform.) With this conclusion the writer is in complete agreement. Davis, Daish and Sawyer also noted that when proper precautions are taken to avoid the hydrolysis of starch and dextrins, maltose is invariably absent from the leaf. They consider that in the plant, the reactions

$$\text{Starch} \rightarrow \text{Dextrins} \rightarrow \text{Maltose} \rightarrow \text{Glucose}$$

proceed under the action of the appropriate enzymes, "liquefying enzymes" (amylase) for the first stage, dextrinase for the second, and maltase for the third. Presumably these enzymes also catalyse the reverse process of synthesis.

A very striking example of the failure of a common plant reaction, when the appropriate mechanism is lacking is given by Davis and Sawyer [1915, p. 357] concerning the enzymes of selective yeasts. The ordinary plant, both

green and parasitic, etc., can quite easily be supplied with carbohydrates as sugar solutions, including maltose and, what is usually the best of all, cane sugar. On the other hand, the yeasts *Saccharomyces anomalus* and *S. exiguus* which do not contain maltase, are unable to assimilate maltose but starve in its presence. Similarly, *Torula monosa*, which does not contain invertase, will starve in the presence of cane sugar.

The apparent anomaly of the sugar-beet and mangold, which though containing maltase and diastase in the green leaf, do not form starch, may be explained by the assumption, that in order for starch to be formed a certain minimum concentration of hexose is necessary. In these plants, before the minimum concentration is reached the hexoses are changed into cane sugar which is translocated to the root. According to Robertson, Irving and Dobson, the root of the sugar-beet contains diastase and maltase but no invertase, whilst the carbohydrate there is cane sugar, both starch and reducing sugars being absent according to Girard [1883, 1884], though Colin [1914] states that small quantities of a reducing sugar are found in the root, varying from 0·178 of the amount of cane sugar in the very young root, to 0·011 of the cane sugar in the older root. Here it would appear that the lack of starch in the root is due to the lack of the raw material, glucose. Such small quantities of glucose as are found there are insufficient to reach the necessary minimal concentration. Also, because of the absence of invertase, glucose cannot be formed by the hydrolysis of the cane sugar.

Hudson and Paine [1914], working with yeast invertase, concluded that the enzyme does not take any part in synthesis, that the equilibrium of the reaction

$$\text{Cane sugar} \rightleftarrows \text{Invert sugar}$$

is not mobile but the change goes to complete hydrolysis, previous workers who stated that they had obtained synthesis of cane sugar having been misled by experimental errors due to the action of hydrochloric acid on laevulose, and other causes. These facts are difficult to reconcile with the current theory of the nature of enzyme action, which cannot be lightly discarded.

The writer wishes to express his profound thanks to Professor J. H. Priestley, to whom the inception of this work is due [see Priestley, 1913, p. 120], and whose kindly help and criticism enabled it to be carried out. His thanks are also due to Mr J. Parkin who very kindly supplied snowdrop leaves in successive years from his home in Cumberland. The experimental work was carried out in the Botanical Department of the University of Leeds with the aid of a grant from the Research Fund of the University.

Summary of Results.

1. The mature green leaf of the snowdrop (*Galanthus nivalis*) contains amylase, dextrinase and invertase, but no maltase or emulsin.

2. The mature green leaf of the onion (*Allium cepa*) contains amylase, maltase, invertase and emulsin, but no dextrinase.

3. The mature green leaf of the leek (*Allium porrum*) contains dextrinase, maltase, invertase and emulsin, but no amylase.

4. The mature green leaf of the dock (*Rumex obtusifolius*) contains amylase, dextrinase, maltase, invertase and emulsin.

5. It is suggested that the failure of the snowdrop, onion and leek to form starch in the mature green leaf is due to the enzyme machinery necessary for starch formation being incomplete in each case.

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CLXXXVIII. THE ACTIVATION OF PANCREATIC JUICE BY ENTEROKINASE.

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Chepovalnikov [1899] was the first to observe that inactive pancreatic juice developed its proteoclastic activity when treated with intestinal juice. Pawlow [1901], as a result of his extensive researches on the subject, came to the conclusion that enterokinase was a specific enzyme, which converted trypsinogen into trypsin. This view of the enzyme nature of enterokinase has been amply confirmed by the work of later investigators, and is now accepted on all hands. However, various hypotheses have been put forward, from time to time, to explain the mechanism of interaction between trypsinogen and enterokinase, though it cannot be said that any one of them has yet been definitely established, and it was in the hope of throwing some more light on this interesting, though complicated, problem that the present work was undertaken.

Methods.

The general technique adopted during the course of the present work was essentially the same as that used by Mellanby and Woolley [1912, 2], and consisted in incubating at a definite temperature a mixture of known quantities of pancreatic juice and enterokinase, and estimating the proteoclastic activity of the fluid from time to time by the milk coagulation method, using 0·1 cc. of the fluid for each estimation.

Pancreatic juice. Fistula-pancreatic juice obtained from dogs by the method of Bayliss and Starling [1902] was used during the course of the present investigation. About 100–150 cc. of pancreatic juice were obtained from each dog and it was always found to be clear and limpid, with a p_H 8·4–8·8. It was also observed that the alkalinity of the fresh juice was due almost entirely to the presence of HCO_3', and not CO_3'', as suggested by previous workers. When collected carefully in sterile tubes, without contamination with intestinal mucosa, and kept at $-1°$ to $-2°$, the pancreatic juice did not develop any appreciable proteoclastic activity, even after three months.

Enterokinase. The second 13 cm. (approximately) of the small intestine of a dog was removed immediately after death, washed thoroughly, and the

lightly scraped mucous membrane then ground up with washed sand and distilled water (two parts mucous membrane to five parts water). After standing overnight under toluene at 0°, the fluid was filtered. The slightly opalescent filtrate was diluted ten times with water, and this clear and almost colourless fluid was used for the activation of pancreatic juice. When kept under toluene at − 1° to − 2°, the fluid retained its enterokinasic properties for several months.

Estimation of the proteoclastic activity of pancreatic juice. A method elaborated by Mellanby and Woolley [1912, 2], depending upon the coagulation of milk, was employed for the estimation of the proteoclastic activity of pancreatic juice. The one great merit of this method is its rapidity, which enables one to follow the course of activation through its successive stages by quick estimations at short intervals.

However, the identity of pancreatic rennin and trypsin being still in dispute, the use of the milk coagulation method as an accurate measure of tryptic activity may be questioned. In this connection it may be pointed out that Mellanby and Woolley [1912, 1] put forward the view that all proteoclastic enzymes coagulate milk in the presence of an adequate amount of calcium ions. Again, Vernon [1913], while claiming to have proved the individuality of pancreatic rennin and trypsin, suggested that the milk coagulating power might be taken as an accurate measure of the tryptic content of pancreatic juice, because the tryptic and rennetic zymogens of pancreatic juice undergo conversion into the respective enzymes at the same time and probably to the same extent. Further evidence in support of the milk coagulation method as an accurate measure of the tryptic activity of a substance, was obtained from the results of some experiments in which the rennetic and proteoclastic powers of a sample of "Trypsin" (dry, yellowish powder—British Drug Houses), were compared. Using 0·1 % and 0·2 % aqueous solutions of this sample of trypsin, their rennetic power was estimated by the milk coagulation method, while their proteoclastic activity was determined in the following manner.

To each tube, containing 15 cc. of 1 % caseinogen solution (Hammarsten's "Casein") at p_H 6·8, varying amounts of the trypsin solution were added and the volume in each case was made up to a definite mark with water. After incubation for 1 hour at 38°, the undigested caseinogen was completely precipitated with $N/10$ alcoholic acetic acid[1] and colloidal ferric hydroxide, and filtered. The clear filtrate was boiled for two minutes to destroy the enzyme, and then an aliquot portion was used for the estimation of total nitrogen, by the micro-Kjeldahl method. The results of one out of a number of similar experiments are plotted in Fig. 1, and the close resemblance between the two curves clearly indicates a corresponding relationship between the proteoclastic activity of trypsin and its rennetic properties.

[1] The amount of acid added in each case was just enough to bring the solution to p_H 4·6, which is the iso-electric point of caseinogen.

It may also be mentioned that, although Mellanby and Woolley's technique of the milk coagulation method was used during this investigation, the results were calculated on the assumption that the time of coagulation of milk (in minutes) is inversely proportional to the amount of enzyme present. (A coagulation time of one minute was taken as corresponding to 100 units of trypsin.) No attempt was made to calculate the results with reference to a "Standard curve," as advocated by Mellanby and Woolley [1912, 2], because (i) the comparative results contained in this paper correspond mainly to quick coagulation experiments, in which the simple relation of "inverse-proportionality" is known to hold good; (ii) the difference between the calculated and the "Standard curve values," even in the case of slow coagulation experiments—while it may make a slight difference in the absolute values of the results—is too small to affect the comparative significance of these experiments; (iii) the "Standard curve" obtained with certain samples of trypsin and milk cannot be accurately employed in other cases where the trypsin and milk do not exactly correspond to the original standard, or where the experimental conditions have been varied. Under such circumstances the use of a "Standard curve" was not considered to be of much practical value.

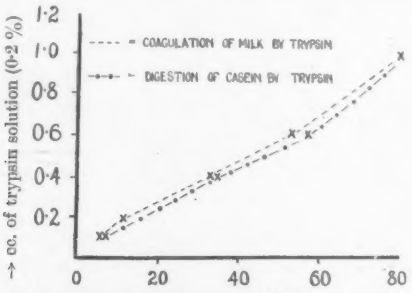

Fig. 1. Comparison between the milk-coagulating and casein-digesting powers of trypsin.

The Activation of Pancreatic Juice by Enterokinase.

The rate and extent of activation of pancreatic juice by enterokinase is greatly influenced by a number of factors which were studied during the course of the present work.

1. *Time.* It was found that the generation of trypsin was a function of the time for which enterokinase reacted with the pancreatic juice (Fig. 2). It was further observed (Fig. 2) that in the initial stages of activation the amount of trypsin liberated per unit time was rather small and later on

increased suddenly and went up to a maximum, after which there was a continual fall, due to a decrease in the amount of the substrate and to the destruction of the free trypsin. That the fall in the activation curve was due partly to a decrease in the amount of substrate, was shown by the fact that a "slowed-down" juice exhibited its original maximum activity on a further addition of inactive pancreatic juice, while the addition of more enterokinase made no difference. It was also noticed that the velocity curve for the activation of pancreatic juice (Fig. 2) was fundamentally different from a typical enzyme-action-curve.

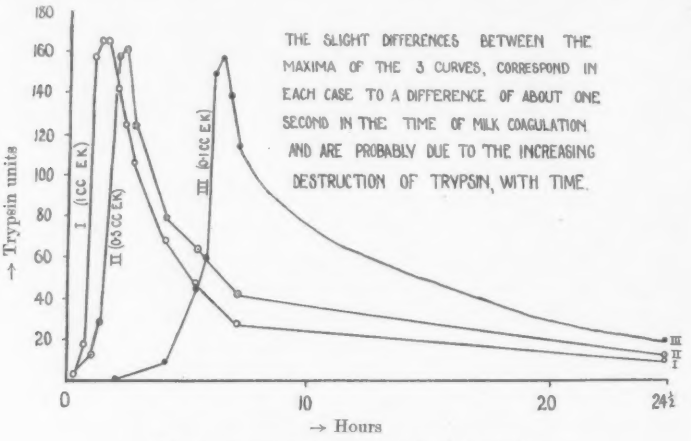

Fig. 2. Activation of pancreatic juice by varying amounts of enterokinase at 38° C.

2. *Enterokinase.* The rate of activation of pancreatic juice was found to be accelerated by increasing the concentration of enterokinase in the mixture but, given the necessary time, the maximum amount of trypsin produced from a definite quantity of pancreatic juice was independent of the initial concentration of enterokinase in the mixture. These two facts, illustrated in Fig. 2, lead one to conclude that enterokinase enters into some sort of a temporary combination with trypsinogen, which combination is in turn broken up with the consequent liberation of trypsin and regeneration of enterokinase.

It was pointed out by Bayliss and Starling [1904, 1905] that the fact that a small amount of enterokinase can activate a very large quantity of pancreatic juice, under suitable conditions, shows that enterokinase is a typical enzyme and that Delezenne's view, regarding the entrance of enterokinase into the composition of trypsin, cannot hold.

It is also obvious that Vernon's view regarding the destructive action of enterokinase on free trypsin is not tenable, since the destruction of trypsin is not increased on increasing the amount of enterokinase in the mixture, even ten times (Fig. 2).

3. *Temperature.* At higher temperatures a marked acceleration in the activation of pancreatic juice and a corresponding increase in the destruction of free trypsin were observed (Fig. 3). It will be noticed that the maximum activity was attained in about one hour at 38°, in about six hours at room temperature, and in more than twelve hours at 3°–5°. The very rapid destruction of free trypsin at higher temperatures is very clearly illustrated by the lower maximum recorded, and the permanent destruction of the whole of the trypsin after 23 hours at 38° (Fig. 3). It may also be mentioned that after incubation of the pancreatic juice-enterokinase mixture for 23 hours at 38° no trace of trypsinogen, enterokinase or trypsin could be detected in the fluid.

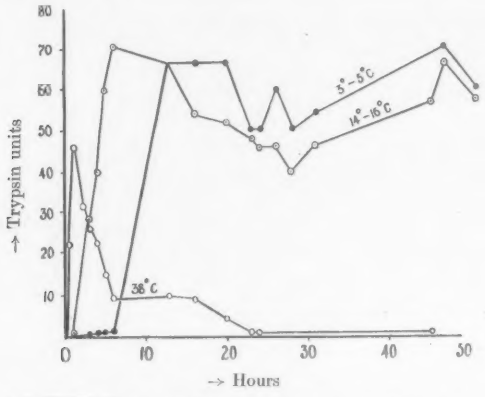

Fig. 3. Activation of pancreatic juice by enterokinase at 38° C., 14°–16° C. and 3°–5° C.

From the above-mentioned considerations it is clear that the activation of pancreatic juice at a comparatively low temperature represents the actual process more exactly than a corresponding activation at a higher temperature, because in the latter case—besides the complications arising out of the simultaneous destruction of trypsin—the velocity of the reaction is too rapid to permit of a thorough study of the intermediate stages of the process. Consequently a large number of the experiments described in this paper were carried out at 3°–5° and some at 1°.

4. *Dilution.* Vernon [1901, 1] observed the rapid destruction of trypsin on increasing the dilution of the active fluid, especially in the presence of alkalies. This phenomenon was observed very often during the course of this work (Fig. 10, Curve D), and in this connection the protective effect of certain colloids was also noticed.

5. *Spontaneous changes in the reaction of the medium during the activation of native pancreatic juice* (Fig. 4). A mixture of native pancreatic juice (p_H 8·8) and enterokinase, on incubation at 38°, invariably became more alkaline (in contrast to the observations of Mellanby and Woolley [1912, 2]). However,

this increase in alkalinity had nothing to do with the interaction between pancreatic juice and enterokinase, since pancreatic juice alone, under the same conditions, showed a similar rise in alkalinity (compare Curves B and D, Fig. 4) without exhibiting any tryptic activity (Curve C).

Again, a mixture of pancreatic juice (previously neutralised with HCl to p_H 5·6) and enterokinase gave the normal type of activation curve (not shown in Fig. 4) without showing any appreciable change in the alkalinity of the medium (Curve G).

These facts show that the rise in alkalinity of pancreatic juice—which is due to the decomposition of bicarbonate present in it, and the consequent expulsion of CO_2—under the conditions mentioned above, does not alter the course of its activation, though it affects the velocity of that reaction, as is shown later on in this paper.

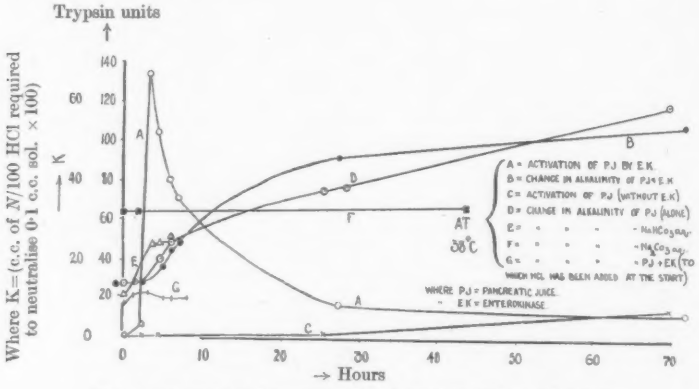

Fig. 4. Changes in the reaction of the medium during activation of pancreatic juice.

6. *Influence of the reaction of the medium on the activation of pancreatic juice.* According to Vernon [1913], a slight alkalinity of the medium is most favourable for the activation of pancreatic juice, while Mellanby and Woolley [1912, 2] maintain that pancreatic juice is best activated in a neutral medium.

In view of these conflicting statements, a series of experiments, extending over a wide range of H-ion concentration were carried out. The general procedure adopted was to incubate suitable mixtures of pancreatic juice and enterokinase containing varying amounts of HCl or Na_2CO_3, and to estimate their tryptic activity at intervals, by the milk coagulation method. In each series of experiments, all conditions, excepting the p_H of the mixtures, were kept constant.

Out of a large number of experiments carried out at 38° and at 3°–5°, a few are illustrated in Figs. 5, 6, 7 and 8, from which the following conclusions are derived:

(i) That native pancreatic juice with a p_H of about 8·8, does not, by any means, present the most favourable conditions for its own activation by enterokinase (Figs. 5, 6, 7 and 8).

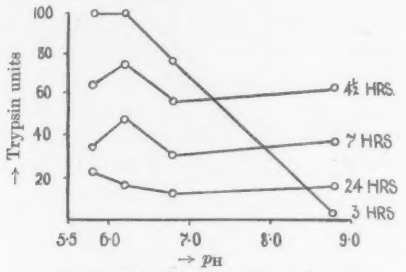

Fig. 5. Effect of p_H on the activation of pancreatic juice by enterokinase at 38° C.

(ii) That the region of quick activation lies between p_H 6·0 and 7·0 (Fig. 7). (*Note.* It was found by a blank experiment that the amount of acid added in the above experiments did not, by itself, coagulate milk even in two hours, though a grainy appearance was imparted to the milk in about half an hour, which, however, never resulted in coagulation. So that the observed acceleration in the activation of pancreatic juice, after treatment with HCl, cannot be ascribed to the direct action of the acid on the coagulation of milk.)

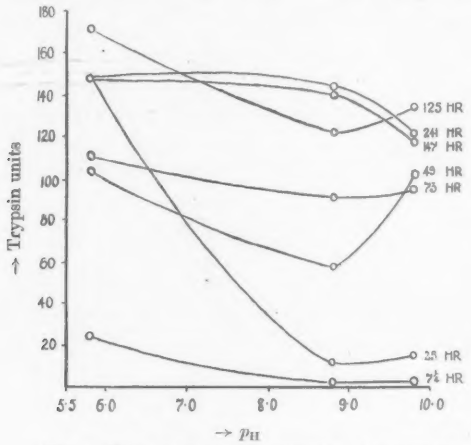

Fig. 6. Effect of p_H on the activation of pancreatic juice by enterokinase at 3°–5° C.

(iii) That between p_H 7·8 and 9·8, the rate of activation is markedly low (Fig. 7).

(iv) That in the vicinity of p_H 10·0, there is another region, in which the rate of activation is comparatively high, though it always falls short of the

corresponding rate of activation in the acid region (Figs. 6 and 7), presumably owing to the destructive action of alkali on free trypsin. The significance of these two regions of high activity, is discussed later (p. 1410).

(v) That on increasing the p_H far beyond 10·0, the rate of activation again falls low (Fig. 7).

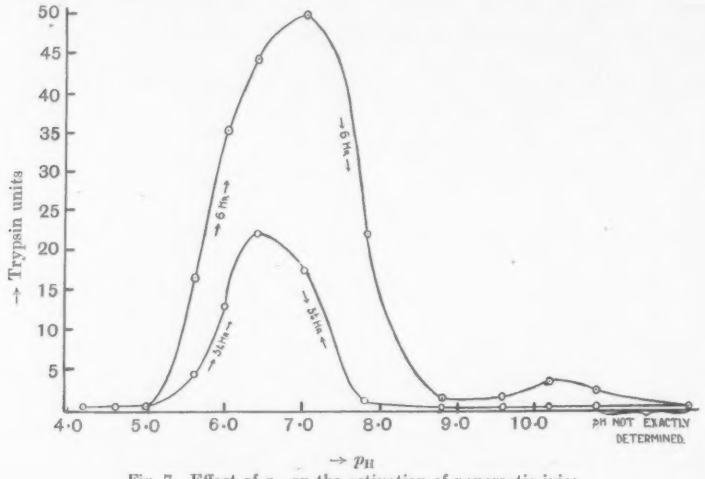

Fig. 7. Effect of p_H on the activation of pancreatic juice by enterokinase at 38° C.

(vi) That on decreasing the p_H below 5·4, the fluid develops no tryptic activity at all. (This was found to be due to the complete destruction of enterokinase and an almost entire destruction of trypsinogen under those conditions.)

(vii) That although a variation in the reaction of the medium affects the rate of activation of pancreatic juice, the maximum activity attained in each case is almost the same, except in very alkaline media, where the maximum is lower, presumably due to the quicker and greater destruction of trypsin (Fig. 8).

7. *Influence of albumin on the activation of pancreatic juice.* The addition of a small quantity of egg albumin to a mixture of pancreatic juice and enterokinase, produces a very marked retardation in the activation process—apparently not unlike the one produced by small quantities of alkali.

Some samples of pancreatic juice were activated by enterokinase at 38°, with and without small quantities of egg albumin, at an initial p_H 6·8. The milk coagulating capacity of the samples was determined at intervals and at the same time the extent of proteolysis in each sample was estimated by Sörensen's formaldehyde-titration method. The milk coagulation results of the above experiments (Fig. 9) show that while pancreatic juice alone attained its maximum activity in about three hours, in the presence of some albumin

the activity attained in that time was almost nil. It is clear, however (Fig. 9), that the extent of proteolysis in both the cases was very much the same, markedly in contrast to the widely divergent results obtained by the milk coagulation method. These observations led the author to conclude that the albumin influenced the activation process, primarily, by sorbing most of the trypsin as soon as it was elaborated, and thus preventing the latter from showing its full capacity for coagulating milk.

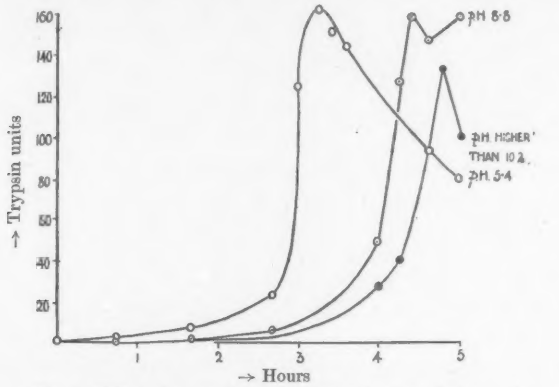

Fig. 8. Effect of p_H on the maximum activation of pancreatic juice by enterokinase at 38° C.

Another interesting point brought out by the above-mentioned observations is that the albumin affords a considerable measure of protection to trypsin against destruction by heat, etc. (Fig. 9).

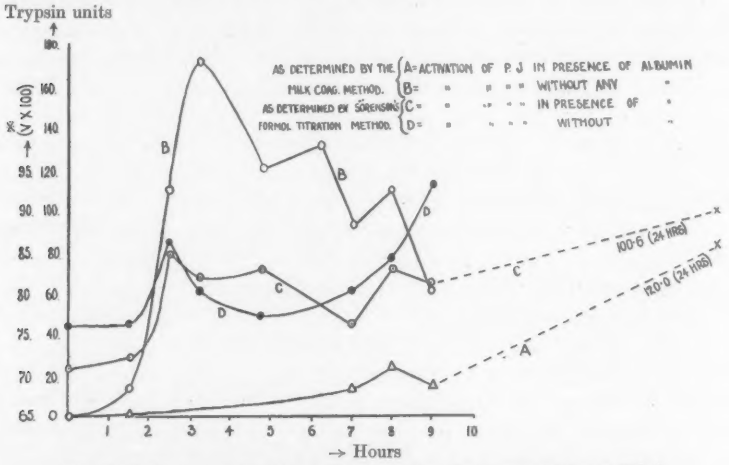
Trypsin units
% (V X 100)
AS DETERMINED BY THE MILK COAG. METHOD.
AS DETERMINED BY SÖRENSEN FORMOL TITRATION METHOD.
A = ACTIVATION OF P J IN PRESENCE OF ALBUMIN
B = " " " " WITHOUT ANY "
C = " " " " IN PRESENCE OF "
D = " " " " WITHOUT "
100·6 (24 HRS)
110·0 (24 HRS)
→ Hours

Fig. 9. Influence of albumin on the activation of pancreatic juice at 38° C.

* V = c.c. $N/200$ NaOH required for formaldehyde titration of 0·4 c.c. of fluid.

Significance of the two maxima in the activation-curve. As has been stated before, a high rate of activation of pancreatic juice was observed in two distinct regions, one on either side of the neutral point. This phenomenon may either be explained by assuming the existence of two separate enzymes, each activating trypsinogen at a different p_H, or it may be ascribed to other causes which may tend to produce two such regions of high activity in the same enzyme. It is suggested that only one enzyme is responsible for both the maxima, obtained at such widely different p_H values, and that these two points correspond to those two positions where the difference between the electrical charges on the enzyme and the substrate is the maximum. The arguments for this view are derived mainly from the work of Loeb on amphoteric colloids [1918, 1922] and that of Northrop [1921, 1922, 1924] on the digestion of proteins by pepsin and trypsin.

It must be understood, however, that although two regions of maximum activity are observed under laboratory conditions, there is no suggestion that the maximum activity in the alkaline region is ever attained *in vivo* under normal conditions, for the simple reason that such a high concentration of OH′ is not maintained in the intestines. Further, in view of the fact that the optimum reaction for the activation of pancreatic juice has been found during the course of this investigation to lie between p_H 6·0 and 7·0, it is of interest to note that the reaction of the chyme is often feebly acid to litmus for a considerable distance along the intestine [Mathews, 1920].

Autocatalysis in the Activation of Trypsinogen.

As has been pointed out already, the velocity of activation of trypsinogen varies very widely under different conditions, though the general form of the activation curve remains unaltered. Again, the shape of the activation-velocity-curve is fundamentally different from that of a typical mono-molecular-reaction curve—a fact which aroused much interest among a number of previous investigators, who explained it in a variety of ways. Mellanby and Woolley [1912, 2], believing the activation of trypsinogen to be a true enzyme action, sought to discover those extra factors which caused the activation curve to deviate so markedly from the course of a typical enzyme-action-curve, but without success.

Having failed to discover any external cause for the peculiar shape of the activation-velocity-curve, Mellanby and Woolley [1912, 2] concluded that the curve represented the true course of the reaction, and that it was not thrown into that form by any disturbing influences.

On the basis of the above conclusion, they put forward a hypothesis to explain the observed facts. They suggested that "Enterokinase is a proteolytic ferment and trypsinogen contains a protein moiety with which the trypsin is combined, the nature of the combination being such that the proteolytic properties of trypsin are masked. The generation of trypsin from trypsinogen by enterokinase is effected by the adsorption of enterokinase by the protein

moiety of trypsinogen, digestion of this protein moiety by enterokinase and the subsequent liberation of trypsin." They further assumed that the adsorption of enterokinase by the protein moiety is a slow process, as compared to the subsequent digestion of the latter, and, therefore, owing to an increase during the process of activation in the proportion of enterokinase, relative to trypsinogen, the adsorption stage is shortened and consequently the activation is accelerated.

Vernon, in a series of papers [1901, 1, 2, 1902, 1913], put forward the view that the activation of pancreatic juice was initiated in the intestines by enterokinase, but it was completed almost entirely by the trypsin produced during the process. Later, Vernon [1914] modified his views and suggested that the trypsin liberated from trypsinogen by enterokinase gradually produces an enzyme—"deuterase"—from a precursor which is present in pancreatic juice, and that this deuterase is mainly responsible for the later stages of the activation process.

In connection with the view at that time held by Vernon regarding the activation of trypsinogen by trypsin, Bayliss and Starling [1904] state that "Although such a process would not be without analogy in other catalytic processes known to chemists, it would be quite a unique case among ferments that a product of a reaction should itself be the catalyser of the reaction. Such a reaction would necessarily proceed with an ever increasing velocity." Bayliss and Starling [1904] tried and failed to activate pancreatic juice with Grübler's powdered "Trypsin" or with a sample of "active" pancreatic extract. Thus they concluded that trypsin had no power of activating inactive pancreatic juice and that the slight activation observed in these cases was due to the small quantity of enterokinase present in the fluid.

As has been mentioned before (p. 1406, Fig. 4), a marked increase in the alkalinity of the juice occurs during the process of activation, so that the question of the acceleration of activation towards the end, due to a partial neutralisation of the alkalinity, does not arise at all. Again, it has been shown in this paper (p. 1408) that the proteins present in pancreatic juice do influence the normal course of activation by adsorbing the freshly produced trypsin and also by protecting the trypsinogen and enterokinase molecules from mutual attacks. However, as is shown in Fig. 9, there is an initial period of slow reaction, even as determined by the Sörensen method, which suggests that the influence of the proteins of pancreatic juice is not wholly responsible for the observed deviation of the activation curve from the path of a typical mono-molecular-reaction curve. Evidently, then, there is yet another factor involved which causes a slow production of trypsin in the earlier stages of activation, and which also accounts for the initial slow rate of proteolysis of the proteins of pancreatic juice (Fig. 9).

The above considerations, coupled with the undisputed enzyme-nature of enterokinase, and supported by the further experimental evidence cited below, have led the author to conclude that the activation of trypsinogen is essentially

an autocatalytic process, and that the activation-velocity-curve represents the actual reaction faithfully and is not a deviated form of a mono-molecular-reaction curve.

In order to get a direct proof of the auto-catalytic nature of the activation of trypsinogen, it was decided to observe if trypsin itself could induce such activation in inactive pancreatic juice. With this object in view, some experiments were conducted, with suitable controls, at 3°–5°. When a mixture of inactive pancreatic juice (2·0 cc.), enterokinase (0·2 cc. of 1 : 10 solution) and water (0·8 cc.) had attained its maximum activity on incubating at 3°–5° for four days, and was fairly steady in its tryptic content, it was used as the source of trypsin (i) in these experiments. The following combinations were kept under toluene, at 3°–5°, and their tryptic activity was determined at suitable intervals:

Serial no.	Pancreatic juice cc.	Other additions	Water cc.
(ii)	1·0	0·3 cc. of (i)	0·2
(iii)	1·0	(i) (Heated for 10 mins. at 60° and cooled) 0·3 cc.	0·2
(iv)	nil	0·3 cc. of (i)	1·2
(v)	1·0	Enterokinase (1 : 100) 0·2 cc.[1]	0·3
(vi)	1·0	Enterokinase (1:100) (Heated for 10 mins. at 60° and cooled) 0·2 cc.	0·3

[1] This is equivalent to the amount of enterokinase present in (ii).

From the results of Exp. (ii) (Curve B, Fig. 10), it is clear that "activated-pancreatic juice" (i) has a marked capacity for activating inactive pancreatic juice. This activating power of "activated-pancreatic juice" (i) may be due either to the trypsin or to the small amount of enterokinase present in it. That the latter factor is not involved is seen very clearly from a comparison of Exps. (ii) and (v) (see Curves B and E, Fig. 10). The fundamental difference between Curves B and E indicates that the activating agent in (ii) is a factor more potent than a corresponding quantity of enterokinase. Again, the great rise in Curve B cannot be ascribed to an increase in the activity of the inoculated fluid (i) itself, since the corresponding control experiments (i) and (iv) (see Curves A and D) show practically no rise at all. Further, the effect (on milk coagulation) of the trypsin present initially in the inoculated fluid, is shown in Curve B by the high position of the curve at the point of origin, and therefore the very rapid rise in Curve B, after 24 hours' incubation, cannot be ascribed to the direct influence of the trypsin present in the inoculated fluid. From the above-mentioned considerations it is concluded that "activated-pancreatic juice" possesses the power of activating further quantities of inactive pancreatic juice, mainly by virtue of its trypsin-content. In other words auto-catalysis during the activation of trypsinogen is a very prominent feature of the process.

It is suggested that the failure of some of the previous investigators [Bayliss and Starling, 1904; Mellanby and Woolley, 1912, 2] to discriminate

between the activation of pancreatic juice by "activated-pancreatic juice" and by enterokinase, may have been due to the comparatively high temperatures employed by them, in which case, the intermediate stages of the two reactions would be difficult to observe, owing to the high velocity of the reactions and the very rapid destruction of trypsin at those temperatures.

Delezenne and Ledebt [1922] observed that "Inactive pancreatic-juice to which a suitable quantity of succus entericus has been added, becomes activated at 0°, and the quantity of trypsin which appears is even greater than at 39°, but the time necessary for the process is very much longer. Further, when maximum activity is attained, it is possible by adding some of this juice, to activate a fresh specimen, and at 0° this activation can be transmitted in

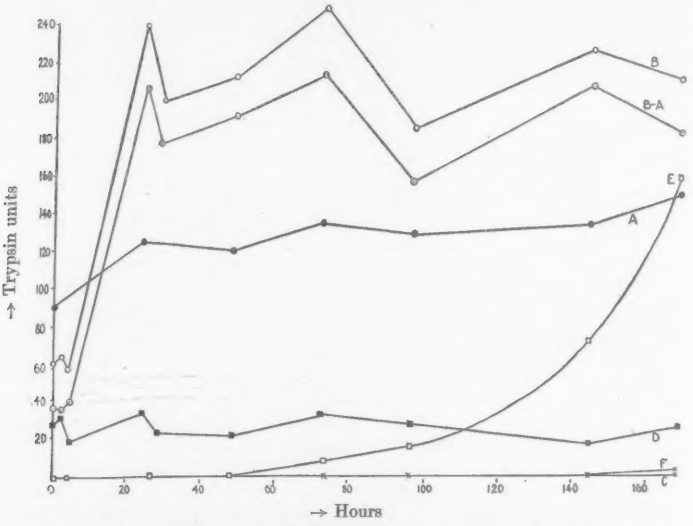

Fig. 10. Activation of trypsinogen by "active trypsin" at 3°–5° C.

series, from juice to juice, indefinitely." In view of the important bearing of Delezenne and Ledebt's observations on the theory of the auto-catalytic activation of trypsinogen, some experiments were carried out by the author at 1°, in which the activating power of fully "activated-pancreatic juice" (p_H 8·8) was successfully transmitted in series to fresh specimens of native pancreatic juice (p_H 8·8) (Fig. 11). At the same time a number of control experiments were conducted at 1°, in which the activating-agent employed was enterokinase in quantities exactly similar to those existing in a certain volume of the "activated-juice" which was used in a corresponding experiment. The results of the above experiments (Fig. 11, Curves B and B′, C and C′) show with an unmistakable precision that the activation of pancreatic juice by "activated-juice" cannot be said to be due entirely or even principally—as suggested by Bayliss and Starling [1904] and Mellanby and Woolley

[1912, 2]—to the activating power possessed by the small amount of enterokinase in it. "Activated-pancreatic juice" has been shown to contain a substance, viz. trypsin, which is a more potent activator of trypsinogen than a corresponding amount of enterokinase, and which is therefore, presumably, the principal activator of trypsinogen *in vivo*.

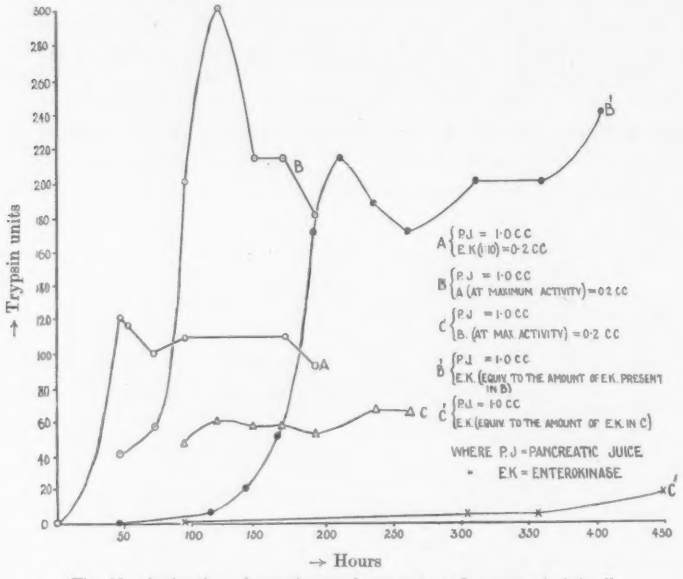
→ Trypsin units
→ Hours
A {P.J = 1·0 C.C E.K.(1/10) = 0·2 C.C
B {P.J = 1·0 C.C A (AT MAXIMUM ACTIVITY) = 0·2 C.C
C {P.J = 1·0 C.C B. (AT MAX. ACTIVITY) = 0·2 C.C
B' {P.J = 1·0 C.C E.K. (EQUIV. TO THE AMOUNT OF E.K. PRESENT IN B)
C' {P.J = 1·0 C.C E.K. (EQUIV. TO THE AMOUNT OF E.K. IN C)
WHERE P.J = PANCREATIC JUICE
E.K. = ENTEROKINASE

Fig. 11. Activation of trypsinogen by "activated-pancreatic juice" and by enterokinase, at 1° C.

It was noticed, however, that the marked alkalinity of native pancreatic juice (p_H 8·8) had a destructive effect on trypsin, so that the activating power of the "activated-juice" was appreciably reduced at the third stage in the series (Fig. 11). Later on, by using partially neutralised pancreatic juice for the purpose, the transmission of the activating power of "activated-juice" could be carried almost undiminished over a much longer series (Fig. 12). However, it must be mentioned that it is not possible to transmit in series the activating power of "activated-juice" indefinitely, as stated by Delezenne and Ledebt [1922], unless means are devised to prevent the slightest destruction of trypsin during the whole course of the experiment. Delezenne and Ledebt's statement, while it is theoretically sound, appears to be difficult to realise in practice on account of the extreme instability of trypsin to a large number of influences like temperature, dilution and variations in the reaction of the medium, etc.

It is a very significant fact to note that, during the transmission in series of the activating power of "activated-pancreatic juice" (Fig. 12), the amount

of enterokinase was considerably reduced at each successive stage, till it might be supposed, for all practical purposes, to have been completely eliminated from the activation system. Thus the activation—particularly in the latter part of the series—may be taken as being due entirely to the activating power

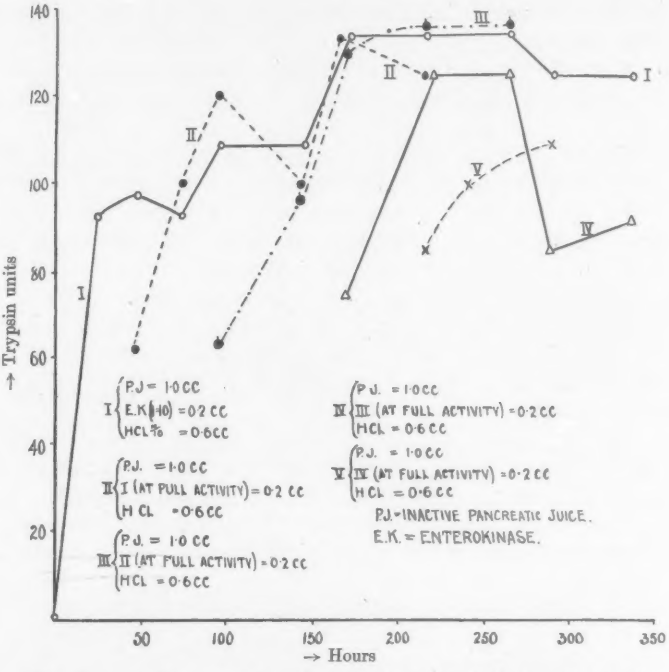
Trypsin units
140
120
100
80
60
40
20
50
100
150
200
250
300
350
→ Hours
I
II
III
IV
V
I { P.J. = 1·0 CC, E.K. (1/10) = 0·2 CC, HCl % = 0·6 CC
II { P.J. = 1·0 CC, I (AT FULL ACTIVITY) = 0·2 CC, H Cl = 0·6 CC
III { P.J. = 1·0 CC, II (AT FULL ACTIVITY) = 0·2 CC, HCl = 0·6 CC
IV { P.J. = 1·0 CC, III (AT FULL ACTIVITY) = 0·2 CC, HCl = 0·6 CC
V { P.J. = 1·0 CC, IV (AT FULL ACTIVITY) = 0·2 CC, HCl = 0·6 CC
P.J. = INACTIVE PANCREATIC JUICE.
E.K. = ENTEROKINASE.

Fig. 12. Activation of trypsinogen in series by "activated-pancreatic juice."

of trypsin. These observations demonstrate that trypsin, under suitable conditions, is capable of activating trypsinogen much faster than, and quite independently of, enterokinase. These facts leave very little doubt about the activation of trypsinogen being mainly an auto-catalytic process, and enterokinase being chiefly an initiator of the reaction.

Conclusions.

From the evidence brought forward in this paper the following general conclusions have been drawn:

(i) That trypsin, as it exists in inactive pancreatic juice, is associated with a protective substance of a colloidal nature, the combination being of such a type as to mask the characteristic properties of trypsin, viz. proteoclastic power, extreme instability to heat, acids and alkalies.

(ii) That enterokinase is a specialised proteoclastic enzyme with a range of activity limited to react with that particular type of grouping only which exists in trypsinogen between a trypsin molecule and its associated protective colloidal covering.

(iii) That enterokinase acts primarily as a stimulus to the activation of trypsinogen, inasmuch as it initiates the reaction, but takes no important part in the later stages of the process.

(iv) That after the initiation of activation by enterokinase, the process is carried to completion, almost entirely by the trypsin produced during the reaction.

(v) That the functions of enterokinase and trypsin—as far as the activation of trypsinogen is concerned—are essentially similar. Both of them activate trypsinogen by digesting the protective part in the trypsinogen molecule, and thus liberating trypsin, which in turn attacks a further quantity of trypsinogen and produces more trypsin. This process, under favourable conditions, continues till the substrate is completely activated.

(vi) That the activation of trypsinogen is essentially an auto-catalytic process, except in the initial stages of the interaction between trypsinogen and enterokinase, where it may presumably be a true enzyme action of the mono-molecular type.

I take this opportunity of recording my best thanks to Professor F. G. Hopkins, F.R.S., for the suggestion of this subject and for constant advice and encouragement. I am also indebted to Dr T. S. Hele for very kindly supplying me with pancreatic juice throughout the course of this investigation.

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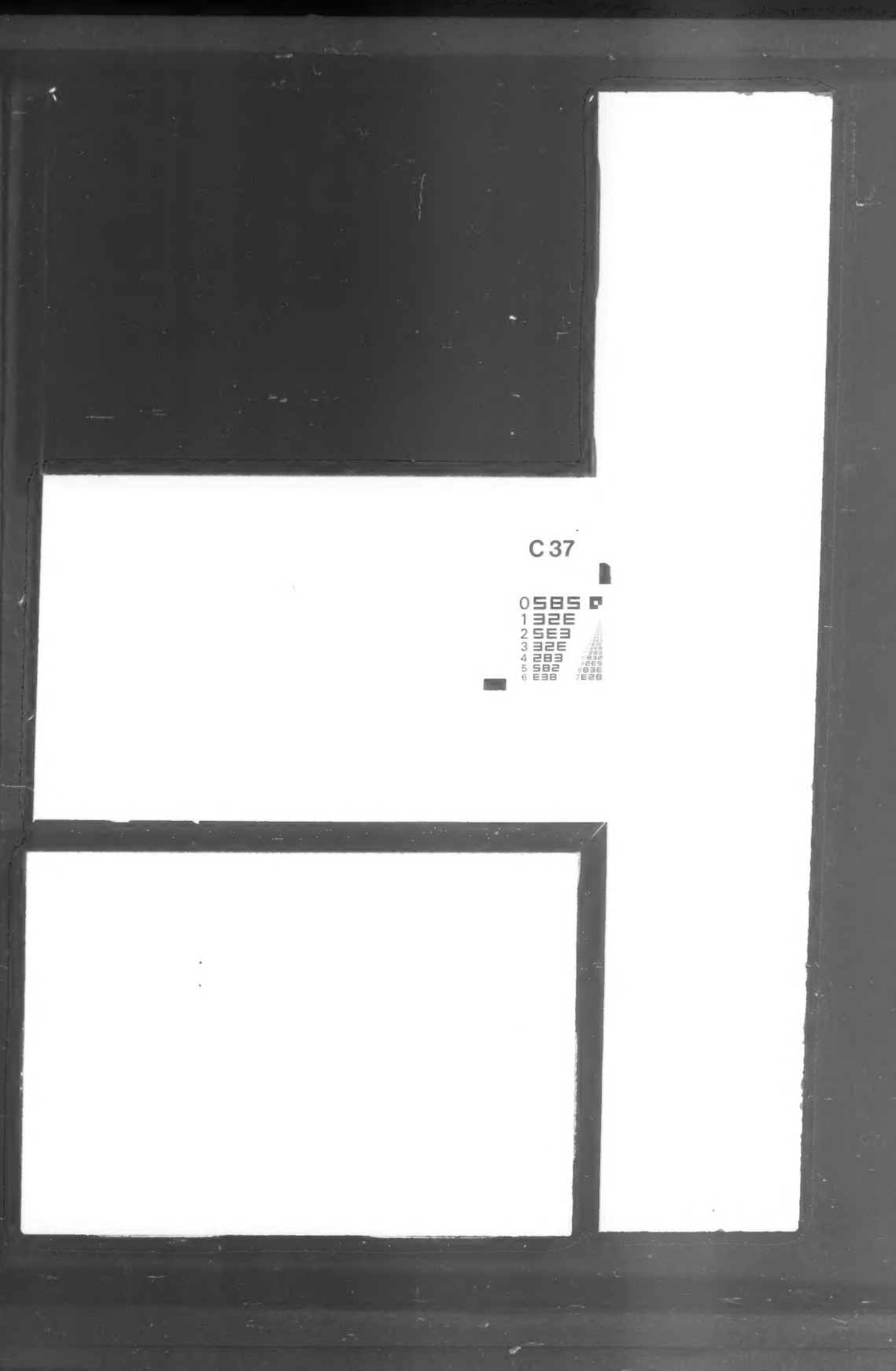
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